THE AMERICAN
JOURNAL OF PHYSIOLOGY

VOL. 121 JANUARY 1, 1938 No. 1

CHEMO-REFLEX CONTROL OF VASCULAR REACTIONS THROUGH THE CAROTID BODY[1]

THEODORE BERNTHAL

From the Department of Physiology, University of Michigan, Ann Arbor

Received for publication April 21, 1937

The existence of a mechanism for chemical regulation of vasomotor reactions through reflexes arising from the carotid body is well established (De Castro, 1926, 1927–28; Heymans, Bouckaert, v. Euler and Dautrebande, 1932a, b). However, relatively little attention has been given to the course of the chemically elicited reactions, their potential strength and their sensitivity. Unfortunately enough, most of the existing data are rendered unsatisfactory as sources of this information by imperfect control of experimental conditions, e.g., contamination of chemically elicited by mechanically elicited reflex reactions because of changing carotid sinus blood pressure, mixture of cardiac and vasomotor events because mean blood pressure variations were used to indicate vasomotor reactions or, in plethysmographic and blood volume flow studies, distortion of reflex by passive vascular reactions due to changing pressure in the blood supply of the peripheral region under investigation.

The present experiments were designed for the study of simple reflex vasomotor reactions which result exclusively from chemical action at the carotid body receptors.

METHOD. Vasomotor activity was followed by recording changes in volume flow of blood in the axillary artery of heparinized dogs narcotized with morphine and urethane. Blood volume flow was recorded by the thermo-electric method of Gesell and Bronk (1926) somewhat modified as to form (Greeley and Greeley, 1930). This apparatus was modified still further by substituting a silver tube for the former glass tube which carried the blood through the water jacket, the improved

[1] Preliminary reports. This Journal **101**: 6. 1932; **109**: 8. 1934. These experiments were supported in part by a grant from the Rockefeller Foundation to Robert Gesell for studies on respiration.

heat conduction reducing the latent period to slightly less than one second.

Chemo-reflex reactions were initiated by inducing chemical changes in the blood supplying the two carotid body regions, vascularly isolated

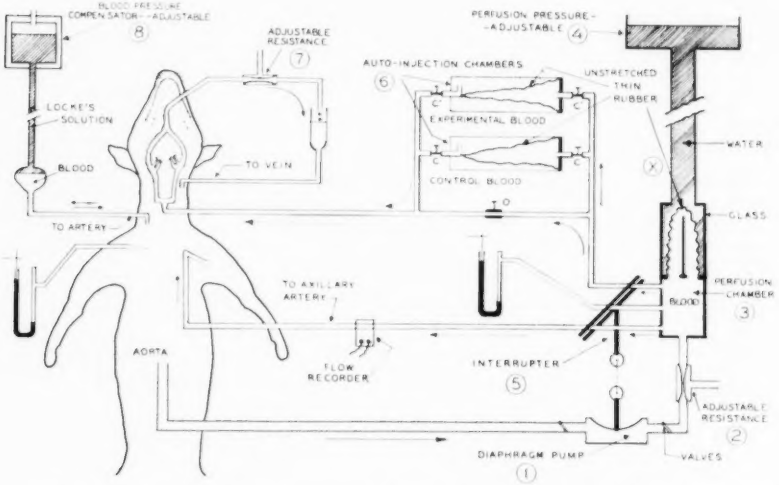


Fig. 1. General schema of apparatus for perfusion of carotid body region and axillary artery. The diaphragm pump, *1*, transfers blood from the aorta into the perfusion chamber, *3*. The resistance, *2*, is so adjusted that the animal is prevented from forcing blood into chamber *3* independently of the pump during temporary rises of aortic blood pressure. *Perfusion pressure is determined by the level of the water reservoir 4* and is transmitted to the blood in chamber *3* through the thin rubber membrane, *X*. Blood is distributed from perfusion chamber *3* to the carotid body and axillary artery perfusion circuits through the separate outlets shown. The outflow from chamber *3* is made pulsatile by the interrupter, *5*. Pump and interrupter are driven by a single motor *and are so timed that the outlets from chamber 3 are blocked during the forward stroke of the pump and are opened only during the back stroke of the pump.*

In the carotid body perfusion circuit, normal blood from chamber *3* can be replaced by blood from the auto-injection chambers, *6*, without interruption of flow or alteration of pressure, by simultaneously closing clamp *O* and opening clamps *C'* or *C*. Endosinusal pressure and rate of flow are adjustable both at pressure source, *4*, and at carotid outflow resistance, *7*.

Both the rate and the stroke amplitude of the pump, *1*, are adjustable while the latter is in operation. Stroke amplitude is continually adjusted in such a way that the blood removed from the animal at the aorta is exactly equal to that returned through the perfusion circuits, deviation from such balance being shown by collapse or inflation of the rubber membrane, *X*.

The systemic blood pressure compensator, *8*, is connected to the cardiac end of any large artery. The whole apparatus is submerged in a constant temperature water bath at 37.5°C.

to prevent chemical agents from reaching other areas. Chemical agents were introduced into the carotid circulation either by injection with hypodermic needle (NaCN, Na_2CO_3, $NaHCO_3$ and lactic acid) or, in the case of blood having altered O_2 and CO_2 tensions, by means of the auto-injection chambers shown in figure 1, *6*. Equilibration of the blood with O_2 and CO_2 was accomplished either in bottles containing known percentages of these gases or in the lungs of the experimental animal itself. In the latter instance the auto-injection chambers were filled directly from the aorta during preliminary intra-tracheal administration of high CO_2 or low O_2 mixtures.

Errors due to the complicating factors mentioned earlier were avoided by the maintenance of constant mean pressure, constant pulse pressure and constant pulse rate in the blood supplying the carotid and axillary arteries (see fig. 1). The perfusion fluid was normal arterial blood, aerated in the lungs of the experimental animal itself. In this respect the perfusion equipment served a purpose similar to that of the donor in crossed circulation experiments, but had the distinct advantage that mechanical as well as chemical factors were under the control of the experimenter.

To avoid abrupt changes in arterial pressure in unperfused regions of the body, especially in the brain, a blood pressure compensator was used (fig. 1, *8*).

Because of the desirability of correlating circulatory with respiratory reactions, the animals were tracheotomized and connected with a rebreathing tank and Hutchinson spirometer for recording tidal air.

Results. *A. The peripheral vasomotor effects of localized anoxemia of the carotid body.* The response was studied in 71 determinations on 12 experimental animals. As figure 2A illustrates, the initial effect of localized anoxemia of the carotid body is an abrupt and intense reflex vasoconstriction peripherally (foreleg). Constriction is maximum in from 25 to 30 seconds, following which there is a rebound to a less intense degree (of constriction) which is maintained throughout the remaining three minutes of anoxemia. The rebound is not a constant feature since the initial degree of vasoconstriction is often maintained throughout the period of anoxemia. Upon readministration of normally aerated blood there is a reversal of the vasoconstrictor reaction which is conspicuously more abrupt than its onset and which is followed by a period of marked vasodilatation, as indicated by the "overshooting" of the blood volume flow. After several minutes the original level of blood volume flow is resumed. That the post-administration overshooting is a reflex phenomenon and is not due to the local vasodilating effects of the preceding curtailment of axillary blood flow is indicated by the fact that respiratory movements (not shown) and often heart rate (to be reported) exhibit analogous changes under similar conditions. It is thus reasonable to assume that

overshooting in recovery is a reflection of a corresponding alteration in the behavior of the carotid body chemoreceptors. A possible explanation of this altered behavior will be considered later in the general discussion.

In the foregoing experiment the cervical vagus nerves were cut *a*, to eliminate the reflex effects of variations of systemic arterial blood pressure acting through the aortic nerves; *b*, to eliminate conflicting chemically elicited reflexes arising from receptors in the distribution of the aortic nerve, and finally *c*, to avoid possible reflex vasomotor changes resulting from varying mechanical conditions at the lungs. Further, in order to minimize secondary changes in the gaseous content of the blood of the general circulation, which might complicate vasomotor reactions by direct action upon the vasomotor centers or by local peripheral effects upon the blood vessels, constant artificial pulmonary ventilation with pneumothorax was used.

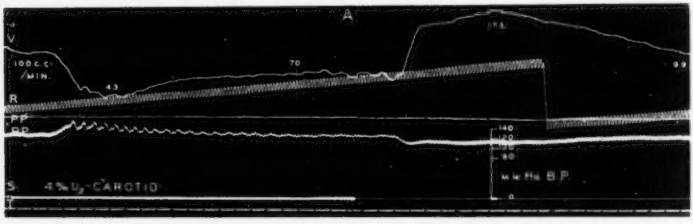


Fig. 2. Effects of anoxemia.

A. Replacement of normal blood in carotid body perfusion circuit with blood previously rendered anoxic in the lungs of the experimental animal during intratracheal administration of 4 per cent O_2. Vagi cut. Constant artificial pulmonary ventilation. *V*, blood volume flow in right axillary artery. *R*, spirometer record. *P.P.*, perfusion pressure with zero 20 mm. above signal. *B.P.*, arterial blood pressure with zero at signal line. *S*, signal. *T*, time in seconds and in 10 second blocks. These abbreviations to be used for all subsequent figures unless otherwise stated.

In experiments in which natural rather than artificial pulmonary ventilation was used, the period of vasoconstriction coincided with increased breathing and the period of post-administration vasodilatation with diminished or diminishing breathing. As a control procedure, simultaneous records were made of the blood flow in the other foreleg which was first denervated. The constancy of the flow in the denervated leg demonstrated the absence of peripheral chemical and mechanical variables in the results described.

When the vagi were left intact results were more variable. Vasoconstriction was less intense and poorly maintained, giving way in some instances to vasodilatation while the carotid body anoxia was still in progress. The post-administration vasodilatation, likewise, was often less marked. Thus the vagus nerve trunk mediates some influence which

inhibits and distorts carotid body chemo-reflex vascular reactions. This influence, we believe, consists of conflicting secondary reflex reactions, further identification of which is deferred to later discussion, the point of significance at the moment being that the findings in vagotomized animals probably do and those with vagi intact definitely do not represent pure carotid body chemo-reflex reactions.

Study of the sensitivity of the chemoreceptors was facilitated by the fact that the O_2 tensions in the perfusing blood could be accurately controlled. In general, blood equilibrated with 18 per cent O_2 mixtures was without effect or caused moderate vasodilatation attended by diminished breathing; but when the O_2 content of the gas mixture was 15 per cent or below, definite vasoconstrictor reactions were obtained. A high degree of differential sensitivity below this level was demonstrated by a definite reflex vasoconstriction upon changing from blood equilibrated with a 12.8 per cent O_2 mixture to blood equilibrated with an 11.4 per cent O_2 mixture—a decrease of 10+ mm. Hg pressure.

B. The effects of sodium cyanide administration localized to the carotid body region. Since cyanide and anoxemia both interfere with the use of O_2, it might be anticipated that they would cause similar responses upon the part of the carotid body receptors and hence similar reflex vasomotor reactions. While the present results are in agreement with this as a general concept, they reveal certain possibly significant differences in the reflex vasomotor responses to cyanide as compared to those of anoxemia.

A typical reflex vasomotor reaction to NaCN is shown in figure 3A. Upon introduction of cyanide into the carotid body perfusion circuit, there is immediate, abrupt and intense vasoconstriction (in the foreleg) which, however, is reversed almost at once. The vasoconstriction is accompanied by increased respiratory movements not shown in this record. After a variable interval, there is a period of moderate but more prolonged vasodilatation, indicated by the increase of blood flow above the preadministration level before recovery is finally completed. This period of later vasodilatation, usually accompanied by diminished respiratory movements, is probably associated with recovery of the carotid body receptors from the effects of the cyanide, in which case it is analogous to the much more conspicuous post-anoxic overshooting.

A marked difference between the effects of cyanidemia and of anoxemia is the rapid lessening of the constriction due to the former as compared to the relatively well maintained vasoconstriction during the latter. That the early diminution of the response to cyanide is due not merely to its being washed away following its injection is indicated by figure 3B. Here the cyanide was mixed with normal arterial blood and administered at a uniform rate and concentration over a period of two minutes by

means of the auto-injection chambers (*6*, fig. 1). The same rapid lessening of the initial degree of vasoconstriction is observed, followed by a full minute during which the volume flow is only slightly diminished in spite of continued contact of the cyanide with the carotid body receptors. Analogous changes are seen in breathing.

Complications which these simple reactions may suffer from secondary effects are illustrated by comparison of figures 3A, 3C and 3D, all from the same experiment and with identical dosages of NaCN. In 3C the inhibiting effect of intact vagi is seen. In 3D the reaction is further

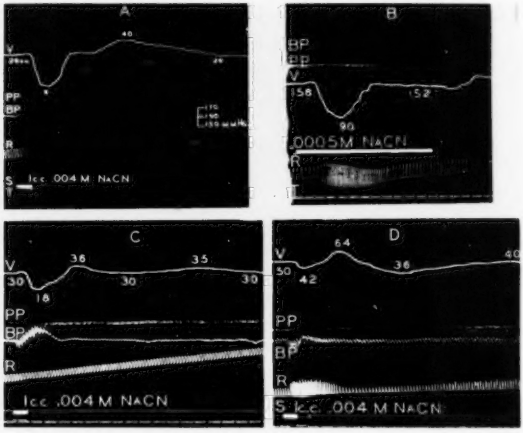


Fig. 3. Effects of sodium cyanide.

A. One cubic centimeter of 0.004 M NaCN injected into carotid body perfusion circuit. Vagi cut. Pneumothorax and artificial ventilation.

B. Continuous carotid perfusion with 0.0005 M NaCN in normal arterial blood. Vagi cut. Natural ventilation.

C. Same experiment and dosage as A, but vagi intact. Artificial ventilation.

D. Same experiment and dosage as A, but with vagi intact and natural ventilation.

In all cases the cyanide reached only the vascularly isolated carotid body region.

modified by the substitution of natural for constant artificial pulmonary ventilation. Whether the modifications in the latter instance originate from chemical (varying aeration of blood supplying aortic nerve endings and vasomotor centers) or mechanical (pulmonary stretch reflex) factors associated with the changing respiratory movements is uncertain, but their demonstration emphasizes the necessity of rigidly controlling mechanical and chemical variables, if reflex vasomotor reactions arising from the carotid body alone are to be studied.

Since the amount and rate of injection of the cyanide and the rate of

blood flow in the carotid perfusion circuit were known, it was possible to calculate the approximate concentration of the cyanide solution bathing the carotid body receptors when a given reaction occurred. The smallest concentration capable of eliciting a recognizable reaction varied considerably in different experiments, being 0.000055 M in one instance and 0.00001 M or less in another.

C. The effects of altered carbon dioxide tension. Because of the potential source of error inherent in the sensitiveness of the carotid body reflex mechanism to variations in O_2 tension, we wish to emphasize the precautions used in controlling that factor. In all CO_2 experiments, the carotid perfusion source alternated between two auto-injection chambers containing blood equilibrated with gas mixtures having O_2 tensions which

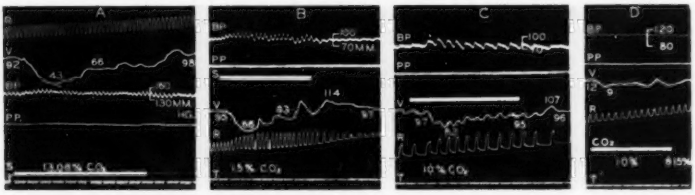


Fig. 4. Effects of excess carbon dioxide.

A. Replacement in carotid body perfusion circuit of blood equilibrated with 5.87 per cent CO_2 + 14.66 per cent O_2 by blood equilibrated with 13.08 per cent CO_2 + 14.40 per cent O_2. Vagi cut. Pneumothorax and constant artificial pulmonary ventilation. For description see text and legend of figure 2A.

B. Same, using bloods equilibrated with 15 per cent CO_2 + 50 per cent O_2 and with 6 per cent CO_2 + 50 per cent O_2. Vagi cut. Natural ventilation.

C. Same, substituting 10 per cent CO_2 + 50 per cent O_2 for 6 per cent CO_2 + 50 per cent O_2.

D. Same, changing from 8.15 per cent CO_2 + 50 per cent O_2 to 10 per cent CO_2 + 50 per cent O_2.

were identical within the limits of error of the Haldane-Henderson method for gas analysis. The CO_2 tensions in the two chambers were the only known variable factors.

The present experiments include 105 determinations in 14 animals. Typical effects of increased CO_2 are shown in figure 4A in which the change in carotid CO_2 tension was from 44 mm. to 98 mm. Hg. The axillary blood flow immediately diminished, after which there was a gradual return toward, but not to, the preadministration level in spite of the maintenance of the increased CO_2 tension. Upon returning to the initial lower CO_2 tension, the axillary blood flow increased for a short period to a rate above the preadministration level, following which recovery occurred. In figure 4B the changes in blood flow are shown to be accompanied by corresponding changes in breathing.

In the majority of instances the CO_2 reactions displayed this tendency toward gradually lessening intensity during continued application of the stimulus, and this, coupled with the less pronounced post-administration vasodilatation, afforded the only conspicuous points of contrast with the reactions to anoxemia. Since the changed gas tensions occurred only in the carotid perfusion circuit, it seems logical to attribute differences in reactions to varying responses of the carotid body receptors themselves. A possible explanation of the differences will be considered later.

To guard against a reduced oxygen transport and impaired oxidations from the excess CO_2, some of the blood equilibrations were performed in an excess of oxygen (50 per cent). The CO_2 reactions persisted in spite of the excess of oxygen (figs. 4C and 4D), but were consistently less intense than those occurring in the presence of more nearly normal oxygen tensions. Unfortunately, most of the experiments were performed in such a way that direct comparison of CO_2 reactions in the presence of normal and of excess O_2 pressures in the same experiment could not be made. In two experiments in which such a comparison was possible, one displayed a most striking reduction of the CO_2 reaction in the presence of the higher O_2 tension while in the other this effect was only slightly in evidence.

The effects of lowering the carotid CO_2 pressure to values approximating zero (3–4 mm. Hg) also were tried. Of a total of 10 administrations of low CO_2, three were without effect, two produced a questionable late vasodilatation and five produced definite and, in some cases, marked reflex vasodilatation which was reversed upon readministration of "normal" (43 mm.) CO_2 pressures.

In view of current differences of opinion concerning the potency of chemo-reflex reactions elicited by increased CO_2, a comparison of the strength of these reactions with more familiar ones seemed indicated. Such a comparison was made in the experiment from which figure 4A was taken. The reaction used for comparison was the mechanically elicited reflex vasoconstriction resulting from complete occlusion of both carotid arteries, this reaction being unusually pronounced in the experiment under consideration. The decreased blood flow shown in figure 4A, and resulting from increased CO_2 at the carotid body, is almost exactly one half the decrease resulting from complete occlusion of both common carotid arteries.

The sensitivity of the reflex response to CO_2 changes varied greatly in different animals, and this is not surprising when one considers the very great risk of interference with both nerve supply and blood supply of the carotid body which adequate "isolation" of the region entails. It is also possible that there are inherent differences in sensitivity among various individuals. The smallest variation in CO_2 tension used was approxi-

mately 15 mm. Hg (alteration between 8.15 per cent and 10.0 per cent CO_2 in the gas mixtures with which the blood was equilibrated). Such changes in the CO_2 tension produced small but definite reflex reactions in blood flow and very slightly increased breathing (see fig. 4D). Inasmuch as these reactions were produced in the presence of excess O_2 pressure, and further, since both of the animals in which the smaller variations in CO_2 pressure were studied displayed only meager reactions to all other types of stimuli as well, it is possible that sensitivity to CO_2 of a much higher order could be demonstrated under favorable conditions.

With reference to the relative effectiveness of similar alterations in O_2 tension and in CO_2 tension in eliciting carotid body reflexes, the former are definitely the more powerful. The results for vasomotor reflexes are thus similar to those of Schmidt (1932) for respiratory reflexes.

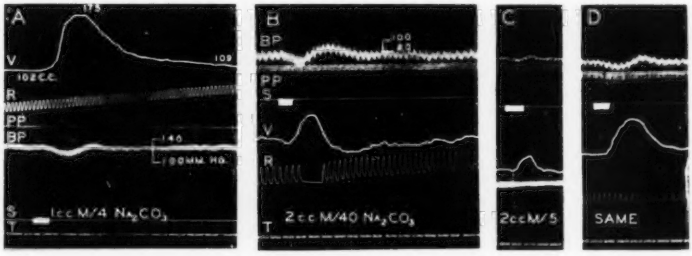


Fig. 5. Effects of sodium carbonate.

A. One cubic centimeter of $\frac{1}{4}$ M Na_2CO_3 injected into carotid body perfusion circuit. Vagi cut. Pneumothorax and artificial ventilation.

B. Two cubic centimeters of $\frac{1}{40}$ M Na_2CO_3. Vagi cut. Natural ventilation.

C. Two cubic centimeters of $\frac{1}{5}$ M Na_2CO_3. Vagi intact. Natural ventilation.

D. Same experiment and dosage as C, but after vagotomy.

D. The effects of sodium carbonate. The reflex vasomotor effects of Na_2CO_3 acting at the carotid body are shown in figure 5. The blood flow in the foreleg rises rapidly to a maximum and subsequently returns smoothly to the preinjection level.[2] In experiments in which natural pulmonary ventilation was allowed (fig. 5B et seq.), it may be observed that the period of vasodilatation is accompanied by depression of pulmonary ventilation. Figure 5B exhibits, in addition, a common varia-

[2] In a preliminary report (Bernthal, 1932) an initial decrease in femoral blood flow was reported to result from Na_2CO_3 administration in the carotid body region. In those experiments, however, injected materials were not confined to the carotid body region, but were carried on into the cerebral circulation. Also, the femoral artery was supplied with blood under the dog's own variable blood pressure. Of these differences in technique, certainly the latter and probably the former were factors in producing the differences in results noted.

tion of the reflex reaction to Na_2CO_3 administration, namely, a temporary dip or undershooting of the volume flow record during recovery. When this undershooting occurs, it is accompanied by increased breathing, and this association suggests that it is the converse of the overshooting and decreased breathing observed after low O_2 and other forms of carotid body stimulation.

The sensitivity of the Na_2CO_3 reaction, as measured by the minimum effective concentration of Na_2CO_3 in the carotid blood stream, varied considerably in different experiments. The most sensitive preparation in the series was still providing definite reactions when the concentration of the injected solution had been reduced to M/320. The calculated concentration of Na_2CO_3 in the carotid blood stream was M/750 (actual dosage 0.65 mgm.).

In the experiment mentioned above, the series of injections was begun with M/10 Na_2CO_3, and the concentration used was progressively lessened. No diminution in reaction was observed until concentrations below M/40 were used, thus indicating that the M/40 solution provided a maximal stimulus for the reflex vasodilator mechanism.

That vagotomy accentuates reflex vasodilator as well as vasoconstrictor reactions is well illustrated by the Na_2CO_3 administrations. Figures 5C and 5D are reactions to identical dosages before and after vagotomy respectively and it is apparent that the duration as well as the intensity of the vasodilatation are increased by the latter procedure.

E. The effects of lactic acid. Following the injection of lactic acid into the carotid body perfusion circuit there was immediate peripheral vasoconstriction followed by recovery during which there was a moderate degree of overshooting (fig. 6A). When natural pulmonary ventilation was allowed, as in figure 6B, the period of vasoconstriction was accompanied by increased breathing and the vasodilatation by diminished breathing. There were differences in the various experiments in the duration of the initial vasoconstriction and in the degree of overshooting during recovery. In all cases, however, the phase of vasoconstriction was much more in evidence than the phase of vasodilatation.

The sensitivity of the lactic acid reaction was tested in only one experiment, in which injections of 2 cc. of M/120 lactic acid repeatedly produced a small vasoconstriction and approximately a 50 per cent increase in tidal air. The dilution of the acid by the flowing blood was determined by calculation to be threefold. Injection of a like quantity of normal saline was without effect.

F. The effects of sodium bicarbonate. As the examples in figure 7 illustrate, the $NaHCO_3$ reactions have two phases,—an initial reflex vasoconstriction of variable but relatively small extent and a subsequent reflex vasodilatation often relatively great in extent and duration. This

diphasic quality merits special attention since it possesses certain features which set the $NaHCO_3$ reactions apart from all others so far studied.

It is true that many of the reactions already described (anoxia, hypercapnia, NaCN, lactic acid) have appeared in two phases consisting of initial vasoconstriction and subsequent dilatation. But in all of these reactions the second or vasodilator phase was very definitely a part of *recovery* from the effects of the chemical agent rather than the direct result of contact between chemical agent and chemoreceptor. Thus, in the case of anoxia, hypercapnia and the cyanide administrations which were performed with the auto-injection chambers (fig. 3B), even when the initial degree of vasoconstriction was poorly maintained during the

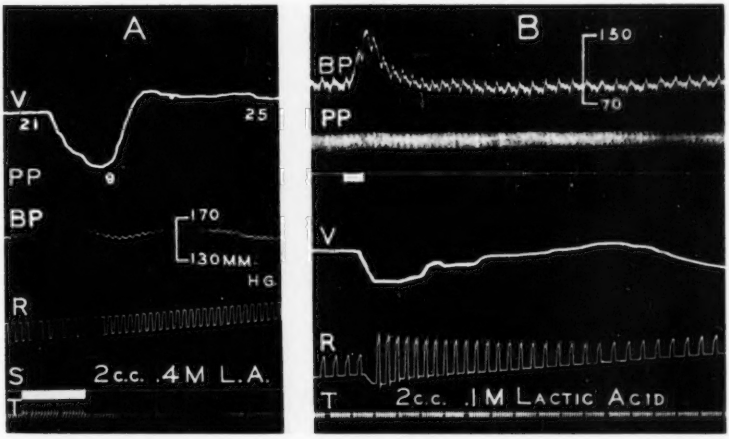


Fig. 6. Effects of lactic acid.

A. Two cubic centimeters of $\frac{1}{10}$ M lactic acid injected into carotid body perfusion circuit. Vagi cut. Pneumothorax and artificial ventilation.

B. Two cubic centimeters of $\frac{1}{10}$ M lactic acid. Vagi cut. Natural ventilation.

chemical administration it was never until *after* the readministration of normal blood to the carotid body region that actual vasodilatation beyond the preadministration level occurred. The second phase of these reactions was thus exclusively associated with the recovery period.

The vasodilator phase of the $NaHCO_3$ reaction, on the other hand, cannot be exclusively associated with recovery processes. In the first place, as study of the injection period, latent period and reaction curve of the responses will reveal (especially figs. 7C and 7D), the phase of vasodilatation had often progressed to a stage such that the blood flow was well above preadministration level at a time when the injected $NaHCO_3$ must still have been present in the carotid regions. Secondly, as illus-

trated by figures 7B and 7D, the magnitude of the phase of vasodilatation as compared with the phase of vasoconstriction was usually too great to allow interpretation of the former as merely recovery from the latter. In figure 7D, for example, the initial effect of the $NaHCO_3$ is represented by only a small vasoconstriction, a transitory hypertension and the acceleration and slightly increased amplitude of one respiratory cycle, and by far the predominating effect is the opposite reaction of vasodilatation, hypotension and apnea. There can be little doubt in this instance that the second phase is a part of the reaction *per se* rather than recovery alone, for it would seem highly improbable that recovery from a reaction could involve changes in the reverse direction several times the magnitude of the reaction itself.

It seems probable, therefore, that the $NaHCO_3$ reactions are unique, among those presented here, in that the reaction itself is diphasic whereas

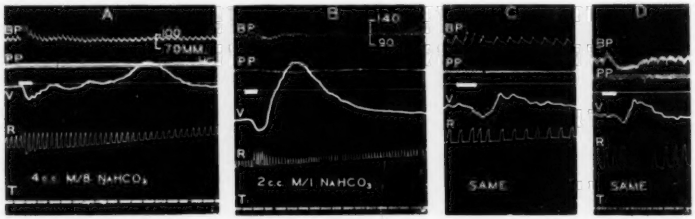


Fig. 7. Effects of sodium bicarbonate.

A. Four cubic centimeters of $\frac{1}{8}$ M $NaHCO_3$ injected into carotid body perfusion circuit. Vagi cut. Natural ventilation.

B. Two cubic centimeters of M $NaHCO_3$. Vagi intact. Natural ventilation.

C. Two cubic centimeters of M $NaHCO_3$. Vagi cut. Natural ventilation.

D. Same, but relatively rapid injection into slowly flowing carotid blood (28 cc./min.).

in all others the reaction itself is monophasic, a second phase appearing only during recovery. The bearing of this characteristic of the $NaHCO_3$ reactions upon a possible mechanism by which chemical stimulation of the carotid body receptors may be accomplished will be discussed in a subsequent section.

Discussion. *Correlation with earlier findings.* Heymans and collaborators (1930, 1932), recording variations in mean arterial blood pressure, established the existence of reflex vasomotor reactions initiated at the carotid body. While the present experiments are concerned primarily with another problem, i.e., with establishing and interpreting the more detailed peripheral pattern of the reflex vasomotor response as it exists when uncomplicated by other circulatory factors, they do, with two exceptions, confirm the gross directional trend of the reactions established by Heymans et al. who observed reflexly increased mean arterial blood

pressure accompanying anoxia, hypercapnia, cyanidemia and hyperacidity (HCl) and reflexly decreased blood pressure upon administration of Na_2CO_3 and other alkaline substances. The present findings are at variance with those of Heymans, Bouckaert, v. Euler and Dautrebande (1932b) as regards the effects of the administration of acid and of $NaHCO_3$. Using relatively strong concentrations of acid (N/2 HCl), these observers obtained inconstant results. In the present series, typical reactions were obtained consistently even though much weaker acid solutions were used (2/5 M to 1/120 M lactic acid). With regard to $NaHCO_3$ administration, Heymans et al. report that reflex stimulation of the vasoconstrictor center did not occur unless the pH of the solution injected was first adjusted to a normal or slightly subnormal level by mixture with CO_2. In the present series of $NaHCO_3$ administrations, the reflex responses were invariably initiated by vasoconstriction even though the pH of the solutions was never adjusted in any way. We suspect that these apparent discrepancies are due to differences in sensitivity of the methods used for detecting vasomotor reactions rather than to actual differences in the responses.

The CO_2 reactions merit separate consideration because of recent differences of belief concerning the responsiveness of the carotid body reflex mechanism to this agent. The original affirmative finding of Heymans, Bouckaert and Dautrebande (1930, respiration) has been contested by Gollwitzer-Meier and Schulte (1931), Mies (1932) and Gollwitzer-Meier (1934) but repeatedly confirmed by others (Schmidt, 1932, respiration and blood pressure; Selladurai and Wright, 1932, respiration; Bernthal, 1934, vasomotor; Heymans, Bouckaert and Samaan, 1935, heart rate; Gayet, Bennati and Quivy, 1935, respiration and blood pressure; Samaan and Stella, 1935, electrical discharge of carotid sinus nerve). The present results add to the already ample support of the original conclusions of Heymans et al. and verify the finding of others that reactions may be elicited by alterations of CO_2 tension *within the physiological range*.

A current question concerning reflex reactions to CO_2 is their importance in comparison to that of reactions resulting from the direct action of CO_2 upon the medullary centers. So far as respiratory reactions are concerned, the evidence indicates that central sensitivity to CO_2 is greater than peripheral chemoceptor sensitivity (Schmidt, 1932; Wright, 1934; Gesell and Moyer, 1937). There being no direct experimental evidence to the contrary, the assumption may be that the same holds true for vasomotor reactions. But, regardless of what the relative sensitiveness of the two mechanisms acting separately may be, we believe that the reflex factor must occupy a significant position in the control of vascular reactions to CO_2 variations in the intact organism. This position is based upon the strength of the CO_2 elicited reactions which these experiments

demonstrate and upon the degree of sensitivity which the reflex mechanism exhibited to CO_2 alterations even under the adverse conditions of these experiments.

The inhibitory effect of the vagi. The repeatedly evident inhibiting and distorting effect of the vagi upon all phases of the reflex vasomotor reactions initiated at the carotid body raises the important question of whether this influence is normally at work in the intact animal, in which case it would tend to nullify the effect of the chemo-reflex reactions, or whether its presence is a peculiarity of the particular set of experimental conditions in use. Hering, Koch and Heymans and collaborators have attributed a similar effect of the vagi upon carotid sinus (mechanically elicited) reflexes to the compensatory action of the aortic nerve pressoreceptors which are exposed to the changes in arterial blood pressure which the sinus reflexes bring about.

In the present experiments, the numerous instances in which the vagus influence was in evidence in spite of successful artificial regulation of the mean arterial blood pressure at a constant level demonstrate that the action of the aortic pressoreceptors alone is not an adequate explanation of the effects noted. If, however, the conception of the aortic nerve compensatory mechanism is broadened to include aortic chemoceptors as well as pressoreceptors, its action may serve to explain the effects noted here. Assuming that this mechanism does adequately explain the inhibitory influence of the vagi upon carotid body reflexes, it follows that this inhibitory influence is peculiar to the experimental conditions used, for under normal circumstances both the carotid and the aortic endings are exposed to identical stimuli and one mechanism would tend to reinforce rather than inhibit the other.

Tonic activity of the chemo-reflex mechanism. When viewed together, the various reactions presented throw light upon certain fundamental problems pertaining to the functional nature and mode of action of the chemo-reflex mechanism as a whole. The first of these is the question of whether or not the carotid body chemo-reflex machinery for vasomotor control is tonically active and, if so, whether the tonic activity is pressor or depressor in its nature. Samaan and Stella (1935) have shown (cat) that there is a "chemical" discharge of moderate intensity in the carotid sinus nerve under the usual conditions of respiration, thus indicating that the chemoceptors themselves are tonically active. This in itself does not prove that the moderate intensity of discharge observed exerts significant effects upon vasomotor tone, nor does it indicate whether the effects are pressor or depressor. But inasmuch as the experimental conditions which were used by Samaan and Stella and by Bogue and Stella (1935) to augment the chemical discharge are included among those which in the present experiments produced reflex vasoconstriction, it is safe to con-

clude that chemoceptor discharge produces reflex vasoconstriction and, therefore, that whatever effect a tonic chemoceptor discharge may have is in a vasoconstrictor or pressor direction.

If we are willing to assume that all of the chemoceptors having vasomotor connections belong to this same functional class, i.e. that their stimulation produces vasoconstriction, then it follows that the only means available for the production of reflex vasodilatation through this source is by inhibition of already active receptors, and the experimental criterion of the degree to which chemo-reflex vasoconstriction initiated at the carotid body is tonically present becomes the extent to which vasodilatation can be produced reflexly through this mechanism when basic or "normal" conditions are the starting point.

There are two types of procedure in the present series which provide examples of primary reflex vasodilatation beginning directly from the normal level of vascular tone. These are the Na_2CO_3 administrations and the experiments in which the CO_2 tension of the carotid body perfusion blood was reduced to subnormal values. If the extent of the reflex vasodilatation is a reliable index of the intensity of the tonic vasoconstriction, then it must be concluded that the tonic vasoconstrictor influence may be of definitely significant proportions, as indicated by figure 5A in which a 71 per cent increase in axillary blood flow resulted from Na_2CO_3 administration.

An alternative interpretation of the vasomotor reactions is that there are two types of chemoceptors, one of which is stimulated to produce vasoconstriction, the other to produce vasodilatation and both of which are quiescent rather than tonically active under basal conditions. However, conditions (subnormal CO_2 pressures) which produced reflex vasodilatation in the present experiments called forth not increased discharge but complete absence of chemoceptor discharge in the experiments of Samaan and Stella, and these results taken together further support the view that there is normally a tonic chemo-reflex vasoconstrictor influence arising from the carotid body.

The fact that denervation of the carotid sinus region produces a rise rather than a fall of arterial pressure does not negate this view, inasmuch as this procedure simultaneously removes the well established and obviously more powerful tonic depressor action of the pressoreceptor mechanism. The phenomenon of simultaneous operation of tonic, mutually antagonistic controlling agencies is not a new one, the mechanism for nervous control of heart rate being a pertinent example.

The identity of the normal chemical stimulus which maintains the tonic vasoconstrictor influence is problematical. The experiments in the present series in which reflex vasodilatation occurred when arterial CO_2 tensions were reduced to subnormal levels support the evidence presented

by Samaan and Stella that the normal CO_2 pressure of the blood may be a factor. It is not possible to say, however, that this is the only factor. The fact that we have sometimes observed reflex vasodilatation when the O_2 percentage of the perfusing blood was raised above normal values leads us to believe that the normal O_2 tension of the blood also plays a part, a logical possibility if one but assumes a high rate of metabolism of the carotid gland.

The nature of chemical activation of chemoceptors. This question has already been considered by Winder (1937) who concluded, as a result of studies with local application of monoiodoacetic acid to the carotid body receptors, that "glycolysis is probably an actual link in setting up the local excitation by anoxia and that the required part of that link is the formation of intermediary or final acid products of glycolysis." Hypercapnic stimulation, on the other hand, according to Winder, may be largely independent of such (glycolytic) processes. If, as these conclusions imply, the acidity of the interior of the chemically sensitive cells is a factor controlling their activity, then it follows that the principles established by Gesell (1929) in his theory of the chemical control of respiration and circulation may be applied to this as well as to the intracranial portions of the chemically sensitive control mechanism.

The present experiments provide a further test of this conception of the control of the carotid body receptors, for the reactions recorded serve as a reasonably reliable index of chemoceptor activity and the several chemical agents employed are all included among those whose effects upon cell C^H have been thoroughly established by Gesell. Comparison of actual and theoretically expected results with such a test in mind reveals that, without exception, the indicated changes of chemoceptor discharge parallel the theoretically co-existent changes in their own acidity.

Anoxia, hypercapnia, cyanidemia and lactic acid excess are all agencies which are believed to cause increased intra-cellular C^H of tissues generally and hence may be expected to do the same for the carotid body chemoceptors. And, in all cases, exposure of the carotid body region to these agencies is accompanied by vasomotor evidence of increased chemoceptor discharge. Na_2CO_3 and hypocapnia, on the other hand, are agents which are believed to cause decreased intra-cellular C^H, and their administration is associated with evidence (reflex vasodilatation) of diminished chemoceptor activity.

A particularly noteworthy example of the apparent parallelism between chemoceptor activity and intra-cellular C^H is to be found in the $NaHCO_3$ records (fig. 7). It will be recalled that in the analysis of these reactions certain peculiarities were noted which led us to believe that both the initial vasoconstriction and the subsequent vasodilatation were direct effects of the presence of $NaHCO_3$, thus making it necessary to differentiate

these from other reactions in which the direct effect of the chemical agent or the reaction *per se*, was monophasic. In terms of chemoceptor activity the $NaHCO_3$ reactions thus signify an initial stimulation followed by depression, both phases of this double reaction occurring as a direct or primary effect of the $NaHCO_3$. Further, in terms of acid control of carotid chemoceptor activity, these reactions would require for their instigation an initial increase of intra-cellular C^H followed by a decrease below pre-administration levels.

Turning to other sources for information concerning the actual effects of $NaHCO_3$ upon intra-cellular C^H, Gesell has shown upon theoretical grounds which are well supported by experimental evidence (Gesell, 1929; Gesell et al., 1930) that intra-cellular C^H will rise following the introduc- of $NaHCO_3$ into the blood stream. The later reaction of cell acidity to $NaHCO_3$ administration may consist simply of a gradual return to a pre-administration level, or it may include a swing into the alkaline side of that level, the determining factor, presumably, being the degree of permeability of the cell membrane to base. Thus, although the late effect of $NaHCO_3$ upon chemoceptor C^H is not strictly predictable, the significant point from the standpoint of the present discussion is that $NaHCO_3$ alone, among the chemical agents we have used, might be expected to cause a primary diphasic response of the receptors such as that which our records have revealed. Thus, in this more complex reaction as well as in the others, a close correspondence between actual and theoretically expected results is apparent.

The conception of acid control of carotid body chemoceptors may be tested further by attempting its use in the logical explanation of other peculiarities in the reflex responses to individual chemical agents. One of these is the very marked degree of post-administration vasodilatation which is manifested by "overshooting" of the volume flow record during recovery from carotid body anoxia. Reasons have been given for believing that this is a true reflex response, and that it is dependent upon a reduction of chemoceptor discharge below the preadministration level of tonic activity. Such a reduction in chemoceptor discharge can be logically explained upon the basis of acid control provided that a shift of chemoceptor C^H into the alkaline side of normal can be assumed to occur during recovery from anoxia. While there is no direct evidence upon which to base such an assumption, such an alkaline shift would be an expected event if the well known principles established by Meyerhof and Hill for muscle may be applied to the carotid body as well. In fact, Gesell (1929) in applying these principles to the interpretation of changes in hydrogen ion concentration of tissues generally, has shown that the alkalinity of the tissues may (theoretically) become greater than normal during recovery from low oxygen administration, the essential phenomena

in the causation of this alkalinity being the liberation of base incidental to the reconversion of the anaerobically formed lactic acid to its precursor state, the base so liberated combining to form additional $NaHCO_3$, thus temporarily increasing the denominator of the $\frac{H_2CO_3}{NaHCO_3}$ ratio until sufficient CO_2 can be accumulated to restore the normal 3/60 relationship. Viewed in this light the reflex "overshooting" which we have observed in the vasomotor response during recovery from carotid body anoxia is not only logically explained, but is the expected result if we may assume an acid control of chemoceptor discharge.

The overshooting which occurs during recovery from cyanide and lactic acid administration may be explained upon the basis of similar reasoning. Likewise the observation that the post-administration overshooting following CO_2 is less pronounced than that following anoxia finds logical explanation if an acid mechanism for chemoceptor control is assumed, since the various characteristics of anaerobic metabolism which are theoretically responsible for post-anoxic tissue alkalinity are largely or wholly non-existent following hypercapnia.

Thus it is apparent that, if chemo-reflex vasomotor reactions are reliable indicators of variations in chemoceptor discharge, a very close correspondence exists between changes in receptor activity and theoretically concurrent changes in their acidity under the varied influences of a large number of chemical agents. Similarly, if reflexly increased or decreased respiratory movements are used as indicators respectively of stimulation and depression of chemoceptors, and if the results are viewed in their light, the same parallelism will be found to exist, situations implying increased chemoceptor acidity being accompanied by evidence of increased chemoceptor discharge and vice versa. We feel, therefore, that these experiments consistently lend very strong support to the conception of acid control of chemoceptor activity.

The fact (Heymans, Bouckaert and Dautrebande, 1931) that the carotid chemoreceptors are stimulated by nicotine, lobeline and other pharmacological agents which do not have known or demonstrable effects upon cell C^H is not a valid objection to the conception of acid control of chemoceptors, for it is not necessary to assume that cell C^H is the *only* stimulus or that cell C^H and other effective agents may not exert their ultimate influence through some common mechanism.

In conclusion, attention is directed to the lack of correspondence between blood C^H and reflex reactions which these results demonstrate. This observation is made in view of the occasional appearance in the literature of the assumption or the implied belief that blood C^H is the factor which controls carotid body chemo-reflex responses.

SUMMARY AND CONCLUSIONS

A method is described which permits the study of reflex vasomotor reactions arising exclusively from chemical changes at the carotid body region and uncomplicated by other circulatory factors. The reactions (foreleg of dog) are described in detail and indications of their sensitiveness given.

Anoxia, cyanide, hypercapnia and lactic acid localized to the carotid body region caused reflex vasoconstriction peripherally (foreleg) during their administration. During the recovery period there was temporary reflex vasodilatation beyond preadministration values.

The vasoconstriction during anoxemia was better maintained and the vasodilatation during recovery more pronounced than were the corresponding phenomena during cyanide administration and during hypercapnia.

Reflex vasomotor reactions were elicited by variations of carbon dioxide tension within the physiological range.

Excessive oxygen tension (375 mm. Hg) in the blood perfusing the carotid body region lessened but did not remove the reflex vasomotor responses to hypercapnia.

Sodium carbonate and hypocapnia localized to the carotid body region caused a simple vasodilatation peripherally.

Sodium bicarbonate localized to the carotid body region caused a diphasic peripheral vasomotor reaction consisting of initial vasoconstriction followed by vasodilatation. Both of these phases were shown to belong to the reaction *per se* as distinguished from recovery, and the significance of this fact in relation to the nature of chemical stimulation of chemoceptors is discussed.

The reflex vasomotor reactions were all markedly accentuated by cutting of the vagus nerves and this phenomenon was attributed to the elimination of the compensatory action of the aortic pressoreceptors and chemoreceptors.

It is tentatively concluded that the carotid body region is the source of a tonic chemo-reflex vasoconstrictor influence and that this is dependent upon the carbon dioxide and oxygen tensions of arterial blood.

The vascular reactions were correlated with the simultaneous reflex respiratory reactions to the same stimuli and a pronounced parallelism was exhibited.

Using reflex vasoconstriction and dilatation as indices respectively of increased and decreased chemoreceptor discharge, a close correspondence was demonstrated between changes in chemoceptor activity and theoretically coexistent changes in chemoceptor acidity. Thus the principles established by Gesell in his theory of the chemical (acid) control of respi-

ration and circulation may be applied to the carotid body as well as to the intra-cranial portions of the chemically sensitive control mechanism.

REFERENCES

BERNTHAL, T. G. This Journal **101:** 6, 1932; Ibid. **109:** 8, 1934.

BOGUE, J. V. AND G. STELLA. J. Physiol. **83:** 459, 1935.

DECASTRO, F. Trav. lab. recherch. biol. Univ. Madrid. **24:** 365, 1926; Ibid. **25:** 331, 1927-28.

GAYET, R., D. BENNATI AND D. QUIVY. Arch. internat. de pharmacodyn. et de therap. **50:** 129, 1935.

GESELL, R. Ergebn. d. Physiol. **28:** 341, 1929.

GESELL, R. AND D. W. BRONK. This Journal **79:** 61, 1926.

GESELL, R., H. KRUEGER, G. GORHAM AND T. BERNTHAL. This Journal **94:** 387, 1930.

GESELL, R. AND C. MOYER. This Journal **119:** 55, 1937.

GOLLWITZER-MEIER, K. AND H. SCHULTE. Pfluger's Arch. **229:** 251, 1931; Ibid. **234:** 342, 1934.

GREELEY, P. O. AND C. E. GREELEY. This Journal **95:** 371, 1930.

HEYMANS, C., J. BOUCKAERT AND L. DAUTREBANDE. Arch. internat. de pharmacodyn. et de therap. **39:** 400, 1930; Ibid. **40:** 54, 1931.

HEYMANS, C., J. BOUCKAERT, U. S. VON EULER AND L. DAUTREBANDE. Compt. rend. Soc. de biol. **109:** 568, 1932a.
Arch. internat. de pharmacodyn. et de therap. **43:** 86, 1932b.

HEYMANS, C., J. BOUCKAERT AND A. SAMAAN. Compt. rend. Soc. de biol. **118:** 1246, 1935.

MIES, H. Ztschr. f. d. ges. exper. Med. **85:** 282, 1932.

SAMAAN, A. AND G. STELLA. J. Physiol. **85:** 309, 1935.

SCHMIDT, C. F. This Journal **102:** 94, 1932.

SELLADURAI, S. AND S. WRIGHT. Quart. J. Exper. Physiol. **22:** 285, 1932.

WINDER, C. V. This Journal **118:** 389, 1937.

WRIGHT, S. Quart. J. Exper. Physiol. **24:** 169, 1934.

THE RESPONSE OF THE SENSORIMOTOR CORTEX TO STIMULATION OF A PERIPHERAL NERVE

S. HOWARD BARTLEY[1] AND PETER HEINBECKER

From the Laboratory of Neurophysiology and Department of Surgery, Washington University School of Medicine, St. Louis, Missouri

Received for publication July 6, 1937

The purpose of this study has been three-fold. First, to compare the action potentials of the sensorimotor cortex with that of the optic whose response to direct stimulation of the optic nerve, and to photic stimulation of the retina, has been subjected to examination, both through manipulation of the stimulus conditions and by recording from different layers in both the normal and strychninized cortex (Bartley, 1933, 1935, 1936; Bishop and O'Leary, 1936; Bartley, O'Leary and Bishop, in press). Second, to throw light on the cortical activity associated with the sensations of pain and touch. These sensations, among others, have been analyzed in terms of the nerve impulse (Heinbecker, Bishop and O'Leary, 1933, 1934; Heinbecker and Bishop, 1934). Through graded direct stimulation activating various groups of fibres in peripheral nerves, and through cutaneous tests under conditions which produce differential loss of sensation, the above authors have identified the nerve fibres responsible for the mediation of the different senses. Third, to compare our results with those of Dusser de Barenne's strychnine technique in delimiting the sensory cortex. Dusser de Barenne (1916) applied weak doses of strychnine to small portions of the cortex, with the result that the animals showed symptoms of spontaneous excitation, paresthesia, hyperesthesia and hyperalgesia. The animals by overt behavior referred these symptoms to specific parts of the body, making possible the correlation of cortical and body areas. By this method, cutaneous sensibility was found to have a degree of bilateral representation, deep sensibility only contralateral. Our experiments have to do with cutaneous sensibility only.

Our animals (cats) were prepared by exposing the anterior portion of the cortex under ether anesthesia in which they were subsequently held during recording, just deeply enough to immobilize them. All potentials were recorded by a surface electrode with the other lead on the skull beside the cranial opening. The potentials were amplified and then observed by use of a cathode-ray oscillograph.

[1] This study was made under a Grant-in-Aid for Neurophysiology to this author by the Rockefeller Foundation.

The experiments consisted in exploring the sensorimotor region of both the normal and strychninized cerebral cortex to find the topographical extent of the effects of stimulation of the ipsolateral and contralateral saphenous nerves by electric shock; and in analyzing these effects by various strengths and rates of repetition of the shocks delivered them. Generally the nerve was first tested directly to determine the stimulus strength necessary to activate the A group, or the A and B groups, or the C group in addition. Such stimuli henceforth shall be termed A, B, and C shocks, respectively.

In the absence of experimental stimulation, the spontaneous waves in the record were generally only of a single kind, a quite continuous succession of waves, the larger ones of which have a frequency of about 50 per second and, on account of their size and rate, simulate most closely the so-called beta rhythm in other preparations and henceforth shall be called that. In some preparations these waves slowly alternated in amplitude, giving the appearance of modulation. There were occasionally under ether anesthesia slow waves of the alpha frequency, which are so prominent in studies on the optic cortex of the rabbit, for example (Bishop and O'Leary, 1936), or are seen at about 7 per second in the occipital cortex of the cat under our conditions, and have been reported (Rempel and Gibbs, 1936) as having a rate of 8 per second in the sensorimotor cortex of the unanesthetized cat and which we record regularly in this area at 10 per second under nembutal anesthesia.

The responses to experimental stimulation were of two general kinds, an immediate wave-complex found at its maximum in the medial half of the postsigmoid gyrus, from where it diminishes to zero in the surrounding gyri, and a delayed and prolonged change in the beta rhythm of the marginal and anterior suprasylvian gyri, as well as the sigmoid, a region much like the total area delimited by Dusser de Barenne (1916) with his strychnine technique. This change in the amplitude of the beta rhythm varies from a doubling to transient almost complete obliteration, depending upon the stimulus variables.

The beta effect. The production of the beta effect (the change experimentally elicited in the beta rhythm) (fig. 1) is dependent upon the strength of stimulation. For example, in one experiment it required either six A or three B shocks or one C shock to produce an increase in the size of the beta waves. Experimental augmentation sometimes lasts only a fraction of a second, whereas at others its duration is several seconds. The same is true for diminution. No definite distinction in the beta effect itself emerged except in the number of shocks taken to produce it, as the shock passed from A to B or C strengths. There was also little correlation between the cortical effects produced and any reflex or other motor activity manifested by the animals. In some, although reflexes

were pronounced, the cortical responses were not as large or as definite as usual. At times, even C shocks did not elicit marked reflexes, doubtless due in part to the depth of anesthesia, and to the circulatory state of the brain. At the same time cortical effects were being obtained. These findings would seem to warrant the assumption that we are recording the sensory activity in the cortex. This supposition is also supported by the fact that strychnine applied to the responding cortex led to no motor manifestations. The delay in appearance of the beta effect varied on several parts of the responding area. The appearance on the suprasylvian gyrus was later than on the lateral part of the postsigmoid. At times a delayed beta effect could be elicited from the anterior sigmoid. *Stimulation of the homolateral saphenous nerve was effective also.*

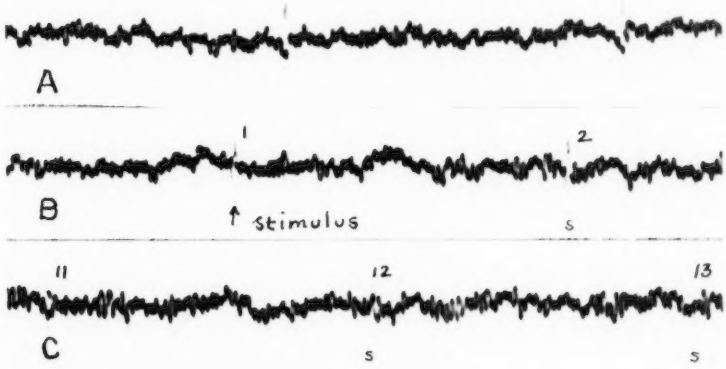


Fig. 1. The beta rhythm (ether anesthesia) under three different conditions. A, with no stimulation. B, at the beginning of stimulation. C, after 12 shocks had been delivered at the rate of 1 per second. Note the augmentation of amplitude from stimulation.

The beta effect is also dependent upon the *rate* of stimulation. This may be illustrated by the following typical experiment. The rate was varied from 110 A shocks per second down to 15. In the highest range, the beta amplitude was increased only if no more than a few shocks were given. At 75 per second, this happened if no more than 15 shocks were delivered. With more, the amplitude was definitely decreased. At 30 per second, augmentation was noted during the first part of continued repetition, diminution during the latter. The threshold rate for diminution with prolonged repetition was about 27 per second, below which rate the change was augmentation if any effect appeared.

A rapid train of shocks elicits a more pronounced beta effect than

shocks given farther apart, no matter how strong they may be. That is to say, in order that the maximum effect be produced, individual shocks must be given close enough together to produce temporal summation. Maximum spatial summation, that is, adding to the number of fibres activated, apparently is not nearly so effective as temporal summation.

If two short trains of shocks were given with varying intervals between, the first train was more effective than the second in producing the beta effect until their separation reached 8 or 10 seconds. For example, with 25 shocks given at the rate of 50 per second, the second train was as effective as the first if 10 seconds were allowed to elapse between the beginnings of each; if only 8 seconds, a noticeable decrease in the second effect was observed; if 3 to 5 seconds, the effect of the first train was not completed and the second shock simply carried on the effect of the first, or possibly augmented it. The beta effect observed in each case was that of augmentation. If the trains were made longer, the effect was diminution of the amplitude of the beta waves. With a 3 to 5 second interval, the second series of shocks prevented full recovery of amplitude, till some time after its completion. If the trains were further separated, the recovery after the second shock series tended to be more rapid.

In those instances in which the beta waves exhibited modulation, stimulation disturbed this alternation of amplitude or increased its rate, and it seemed to depend upon where in the cycle the first shock in a series was given whether or not an immediate beta effect was elicited, and to some extent what the resulting nature of the beta effect would be. By an immediate effect is meant one following the first shock in a slow series, or one following a few rapidly given shocks. The irregularity of the spontaneous modulation of the beta waves (spindles) prevented accurate timing of our stimulation with reference to them.

The immediate specific response. The immediate specific response consists of a short diphasic wave (hereafter the *xy* wave), the first phase surface positive (*x* phase), starting about 10 sigmas after the beginning of the shock. The second phase (*y* phase) is more pronounced and its peak appears at about 20 to 30 sigmas after the shock. It is never more than two or three times the height of the beta rhythm, often being so nearly the same size as to be difficult to distinguish. A small immediate effect can be elicited by stimulation of the ipsilateral saphenous as well as the contralateral. Occasionally, the wave is superimposed on a much broader one (*z* wave) which originates in the positivity just mentioned and quickly becomes negative. The slow ascending limb ends at about 140 sigmas after the stimulus, frequently in a slight positivity. The diagram in figure 2 will make clear its typical characteristics. This broad wave and the short one are not always present and absent together. That is, what augments the one does not necessarily augment the other. More often,

the short one is present without the other, although the reverse is sometimes true. Both can be manipulated by stimulus strength.

The amplitude of the *xyz* complex is not so great as the typical response of the rabbit optic cortex. The *xy* wave often varies in size from shock to shock, perhaps partly due to its combination in the record with the beta. However, at times it has been observed to build up during the first two or three shocks (at 1 per sec.) and then wane during the next three or four, becoming very small. Strong shocks which stimulated the C fibres as well as the others, produced response waves which were lower and more prolonged than did weaker shocks.

The response has been further studied by use of both paired shocks and continuous repetition. The second shock of a pair elicits a response down to the point at which it is delivered before the response to the first has been completed. Frequently, as this point is approached, the response to the second becomes smaller and smaller. Due to the existence of the beta rhythm, it is difficult to tell what the very minimum effective separation

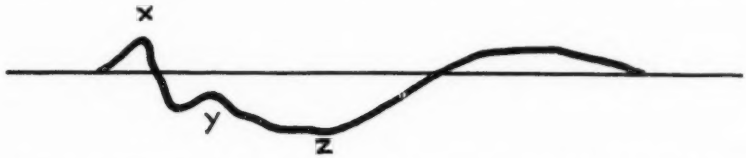


Fig. 2. A diagram of the immediate specific response of the sensorimotor cortex to brief electrical stimulation of the saphenous nerve with animal under ether anesthesia.

can be. However, it is certain that it is only a few milliseconds and thus of an entirely different order from that found in the optic cortex (Bartley, 1936), where the second shock begins to be effective only after an interval of 80 or more milliseconds. Repetitive shocks produce identifiable *xy* waves until the rate reaches about 60 per second, the shock interval being similar to the minimal effective one for paired shocks. In both cases this absolute minimal interval becomes indeterminate in the record for the same reasons.

In previous experiments (Heinbecker, Bishop and O'Leary, *l.c.*) on human subjects, when peripheral nerves were stimulated directly, it was found that increasing the stimulus strength within the range of one type of sensation did not produce perceptibly different qualities of sensation. This did not apply, at least in one respect, to changes of frequency. For touch, individual shocks, up to 30 per second or slightly more, could be detected if the attention was fixed on them. At 60 per second, the sensation had become smooth. That is to say, at slightly above threshold intensity, the repetitive character of the stimulus could be recognized at

30 but not at 60 per second, and this persisted throughout a range of intensities above threshold. Painful stimuli likewise gave an experience with the quality of roughness at 30 per second, which changed to smoothness upon approaching 60 per second. Painful stimuli were also more tolerable at 60 per second and above than at 30 per second. This is quite parallel to the facts of visual sensation (flicker) with intermittent stimulation, in which the fusion frequency is supposed to be determined in the eye. The representation of fusion for the cutaneous senses was seen in our cortical records, but we do not know the part of the central nervous system immediately responsible. The phenomena which might represent this fusion were seen in both the immediate response complex and the associated beta activity. For example, we frequently noted that the amplitude, or area, of the first complex remained constant at any frequency of stimulation up to about 30 per second, above which it became variable. At frequencies above 60, it was unrecognizable. The beta effect up to rates of 30 per second tended to be that of augmentation in amplitude, and possibly in frequency; above that, diminution quickly set in if more than a few shocks were administered.

This is quite similar, in some respects, to the behavior of the immediate response of the optic cortex, in that individual stimuli tend to synchronize the elements responsible for the waves, but if very rapid *trains* of stimuli are delivered, instead, the opposite is true. That is, when isolated shocks or flashes are used, the waves of the immediate responses are large; when very rapid trains of shocks, the waves dwindle to the vanishing point. The difference between the immediate response and the beta waves, of course, is that the latter exist when no experimental stimuli are arriving at the cortex. We can say, then, that whether the elements responsible for the two types of phenomena we have been comparing are active continually or only at the time of experimental stimulation, or whether they are active all the time but in such a temporal sequence as not to be recorded, varied spacing of stimulation tends to bring about the same type of change in the activity, namely, either more or less synchronization. The magnitude of the result is dependent on the factor operating in the circuit at the moment, such as possibly the amount of stimulation coming from elsewhere in the periphery. This factor, for example, would not operate so directly and effectively in vision as here, for the whole input into the optic nerve, the channel representing the whole visual periphery can be controlled.

In order to account for the existence and nature of sensation resulting from very high frequency stimulation, it is to be presumed that the elements responsible for the *xy* wave can follow frequencies higher than 60 per second. Adrian has shown that the touch end organs in the cat's paw can fire off at a rate of 150 per second when a "physiological" stimulus is used. This would leave an interval of $6\frac{2}{3}$ milliseconds between dis-

charges, a much shorter time than the duration of the complete *xy* wave, or even shorter than its latency. If the *xy* wave represents a series of events in a series of elements, the activity of one the necessary precursor of the next, then a second *xy* wave may be instituted before the completion of the first, by reactivating the same elements in the same order. Up to frequencies which produce sensory fusion or smoothness, succeeding shocks apparently activate the same elements, it being only beyond the fusion point that alternation may possibly take place. This is in contrast to the behavior of the optic cortex, where alternation seems to begin below fusion frequency for repeated light flashes. This difference, if it be true, is important to note, although its full significance is not yet apparent.

Effect of strychnine and different degrees of anesthesia on the immediate response. The implication that the *xyz* complex and the beta effects are more or less independent and involve possibly different elements is strengthened by the use of strychnine and the manipulation of the depth of anesthesia. When strychnine is applied to the surface of the responding cortex, the *xy* wave becomes much enlarged. This enhancement begins by hugh increases in amplitude, leaving latency and duration unchanged. Generally, the *y* phase becomes much more enlarged than the *x*, which may remain about its original size. Before the enhancement of the response, spontaneous waves, similar to it, appear, and often at a fairly regular rate, persisting under conditions in which the response can no longer be elicited.

After once becoming increased in amplitude, the *y* wave undergoes further change. It becomes prolonged, comparing no longer to the original *y* wave but more nearly to that of the negative phase of the normal *z* wave. After this, the single peak may be replaced by two, the separation of which varies greatly from time to time, the one appearing earlier than did the original peak and the other later than it. Figure 3 shows a series of strychnine responses taken at fairly regular intervals over a period of about an hour. First of all, it is obvious that the latency of the response as well as the time of appearance of the one or two peaks fluctuates. The amplitude of the wave in general varies somewhat inversely as the separation of the peaks. The peaks vary more or less independently in their height but relatively not so much as the general size of the response. The last part of the series in figure 3 shows the large wave in the response complex to be very late, foreshortened and preceded by several ripples which possibly correspond to some much larger components of the response earlier in the hour. One of the forerunners to the ripples just mentioned is the much reduced first peak of waves 32 and 35. By the time that the last records were taken, the response had become hard to elicit; several shocks had to be given before the response would appear, and after a few more it again failed. The whole behavior of the response complex indi-

cates that strychnine shifts both the total response, sometimes making it very late, and allows different components to stand out in the record from time to time.

Many observations show that the strychnine response may have a latency much longer than any shown in the series in figure 3. It is as if the spontaneous wave were about to appear and a shock were given at the proper moment to hasten it, for it must not be forgotten that all the time we were obtaining the responses to stimulation, occasional spontaneous waves were appearing.

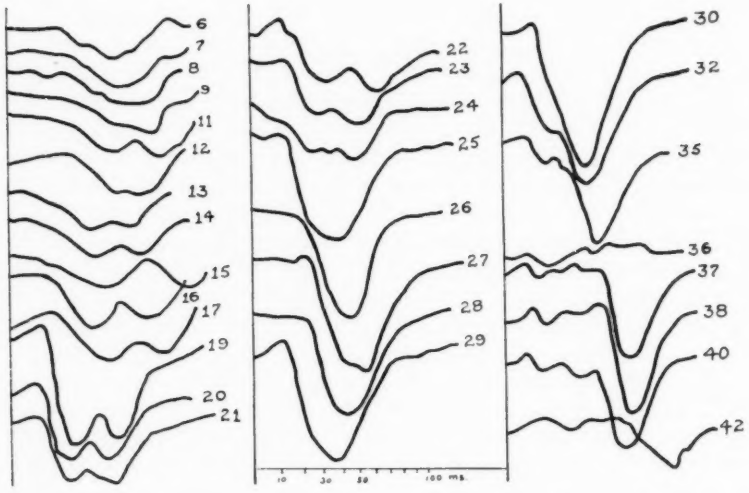


Fig. 3. A series of immediate specific responses under the influence of strychnine, recorded at fairly regular intervals over a period of an hour. Note the increase in amplitude, the double troughs of waves 16–23, the variation in latency, its apparent final lengthening though augmentation of late components of the response with the diminution of the early ones would similarly modify the response.

Late in the strychninized state, response to stimulation was hard to obtain, and several shocks had to be given before we could get a response to each of several consecutive shocks. This same phenomenon of facilitation appeared in a little different situation, that is, in cases in which paired shocks were delivered 35 to 70 sigmas apart, once every 1 to $1\frac{1}{2}$ seconds. At the onset, the first of the pair elicited a response, and the second shock followed too soon to be effective. After a while, the first shock failed and then the response followed the second shock, that is, the two shocks together were able to elicit it. Before the first shock completely failed, it elicited a small response; at which time a response began to follow the

the second shock. Varying this spacing manipulated the latency of the response following the second shock. For example, when the shocks were 38 sigmas apart, the implicit time was 98–100 sigmas; when 50 sigmas, the implicit time was 78 to 80 sigmas; and when 66 sigmas, the implicit time was 70 to 72 sigmas. Undoubtedly these values would shift under other degrees of strychninization.

During strychninization the beta rhythm is left unchanged. Increasing the depth of anesthesia to the point of stopping respiration leaves the spontaneous strychnine waves as large as ever, but reduces the beta rhythm amplitude, which returns to its former size upon lightening the anesthesia again.

General discussion. With the use of specific accurately timed stimulation, certain phenomena are revealed that might escape the observer noting the effects of incidental and random environmental changes upon cortical potentials. Both our immediate specific response and the beta effect seem to be instances of this kind. Cortical changes brought about by gross environmental changes noted by various investigators have been mostly confined to the alpha rhythm. On this account, the results reported here cannot be strictly compared to the findings of many who have been studying cortical potentials, but are most immediately useful in the way indicated in the introductory remarks (*q.v.*).

At the present time, none but the more superficial similarities and differences of the sensorimotor and optic cortices have been outlined, although the experiments have enabled other comparisons of them. It is significant that the spontaneous pattern of the two is dissimilar. The sensorimotor cortex, under our conditions (ether anesthesia), normally lacks the extremely large slow waves (the alpha rhythm). The optic cortex exhibits both the alpha rhythm and a repetition of the fourth component of the immediate response complex at the rate of the alpha rhythm (Bishop and O'Leary, 1936), but the immediate response complex in the sensorimotor cortex does not tend to repeat as far as we can see. However, the response is so little higher than the spontaneous beta waves that it would be difficult at times to tell if it did repeat. The response of the sensorimotor (at least the *xy* wave) to the second of two maximal A stimuli can follow very few sigmas after the first. In fact, the two can overlap. In the optic cortex, the second response will not occur till considerable time has elapsed, generally at least 80 milliseconds. In terms of the immediate response complex, the second response will not occur until the beginning of the fourth component of the first. After the time for the fourth component, the second response again fails to appear or is small. The alpha rhythm signalizes a waxing and waning in the excitability of the optic pathway and there are indications that the seat of this excitability rhythm is in the thalamus.

The immediate responses of the two cortices elicited by experimental stimulation belong to a different set of events from those characterizing the fast waves (beta rhythm), as judged by the fact that what manipulates the one may fail to alter the other. On both cortices strychnine prevents the necessary facilitation to produce a response to a second stimulus at the normal minimal interval, so that a second shock will be effective only after a much greater interval. In the optic cortex this is often of the order of a second; in the sensorimotor, not quite so long. However, the actual order in either case is dependent upon the amount of strychninization, complete blocking being possible, before which long intervals have been observed. Somewhere within this interval, in the strychninized cortex, two shocks may summate to produce a single response.

Our findings also bear upon those of Dusser de Barenne who found that for any part of the body surface there was not only a focal area on the cerebral cortex, but that certain areas around it also participated. Our immediate specific response seems to represent the activity of the cortical focus for the part stimulated. The beta effect, though somewhat more elusive in character than the symptoms which he observed, bears out the widespread cortical connection with specific regions of the body. He also found bilateral representation of cutaneous sensibility, another observation which we were able to confirm in the findings of both specific response and beta effect.

With stimulation of the saphenous, that is, the stimulation of a portion of the hind limb, we noted the beta effect in areas Dusser de Barenne had shown to be focal areas for other parts of the body. The part he assigned to the head (anterior sigmoid) was our most inert area; in it, the beta effect, if it appeared at all, was much delayed.

SUMMARY

The observable cortical effects from stimulation of the fibre groups in the saphenous nerve of the cat under ether anesthesia are described.

They consist of an immediate response more or less specific in nature and an effect on the beta rhythm. The former is relatively localized, the latter is found over much wider area.

A correlation between the sensory effects obtained by stimulation of the touch and pain fibers and the characteristics of the recorded cortical activity is presented.

By the use of strychnine applied to the cortical surface and by varying the depth of anesthesia the relative independence of the immediate response and the beta effect is demonstrable.

REFERENCES

BARTLEY, S. H. This Journal **103:** 203, 1933; **110:** 666, 1935; J. Cell. and Comp. Physiol. **8:** 41, 1936.

BARTLEY, S. H., J. O'LEARY AND G. H. BISHOP. This Journal (in press).
BISHOP, G. H. AND J. O'LEARY. This Journal **117:** 292, 1936.
DUSSER DE BARENNE, J. G. Quart. J. Exper. Physiol. **10:** 169, 1916.
HEINBECKER, P., G. H. BISHOP AND J. O'LEARY. Arch. Neur. Psychiat. **31:** 34, 1934.
HEINBECKER, P. AND G. H. BISHOP. Proc. Assoc. Res. in Nerv. and Ment. Dis. **15:** 226, 1934.
REMPEL, B. AND E. L. GIBBS. Science **84:** 334, 1936.

REFLEX RESPONSES OF THE NICTITATING MEMBRANE AND THE BLOOD PRESSURE TO DISTENTION OF THE BLADDER AND RECTUM

A. L. WATKINS

From the Department of Anatomy and the Department of Medicine of the Harvard Medical School, and the Medical Clinic of the Peter Bent Brigham Hospital, Boston

Received for publication July 6, 1937

The presence of reflex changes of blood pressure (b.p.) upon adequate stimulation of abdominal viscera has been recognized for some time. Sherrington (12) in 1899 showed that distention of the gall bladder and ureter produced a rise in b.p. Changes both in heart rate and in the electrocardiogram produced by distending the gall bladder, intestines and urinary bladder in animals have been reported by various authors (4, 9, 11, etc.). Falls in b.p. were recorded following the distention of intestines in dogs by Morin and Vial (7) in 1934. Frequent clinical observations have also been made by urologists of the fall in b.p. subsequent to sudden emptying of chronically distended bladders (3, 5, and others). Recently Talaat (13) noted that in dogs in which he induced high bladder pressures the b.p. rose 15 to 30 mm. Hg. In addition it has been shown by Rosenblueth and Schwartz (10) and Acheson, Rosenblueth and Partington (1) that reflexes of the nictitating membrane (n.m.), as well as of the cardiovascular system, may be evoked by electrical stimulation of visceral afferent nerves.

In the literature cited, however, there were no studies on the quantitative relationships between visceral pressure and volume and the reflex rise in b.p., nor on the effect on the n.m. of distention of the hollow viscera. The purpose of this paper is to present some observations on these problems as related to the bladder, rectum and sphincter ani.

Methods. Male and non-pregnant female cats were used during April and May, 1937. After etherization, urethane (1–1.5 grams per kgm.) was injected intravenously. In control experiments other anesthetics (sodium pentobarbital and dial (Ciba) 0.7 cc. per kgm., intraperitoneally) or decerebrate preparations were used. In certain cases the vagi and depressor nerves were cut, carotid sinuses denervated, and the adrenals tied.

The b.p. was recorded from a carotid artery by means of a mercury

manometer and in all cases was higher than 120 mm. Hg. Isotonic responses of the n.m. were recorded by a lever exerting a tension of approximately 2 grams and affording a 10 to 15 fold magnification. The respiratory movements were recorded by connecting a tracheal cannula to a writing tambour through a T-tube, the other branch of which was open. The bladder was cannulated at its base with a glass cannula extending through a T-tube to a mercury manometer and to either a 150 cc. graduated syringe or to a reservoir bottle of warm saline which could be raised to desired heights and pressure accordingly produced within the bladder. Bladder volume was measured by withdrawing the contents into a graduated cylinder with care to empty the bladder completely. The rectum was distended by the same apparatus, a rubber balloon being tied on to the end of the cannula and inserted into the rectum. The pressure recorded in this case was not that applied to the rectum, as no allowance was made for the resistance of the balloon. The rectum, however, was dilated to a maximal diameter of 4 to 6 cm. In a number of experiments the bladder was displaced extra-abdominally and kept moist at a temperature of 32 to 37°C. in an atmosphere of steam and with frequent applications of warm saline. In some male cats a fine glass cannula was inserted through the penis a short distance and tied and the urethra ligated at the base of the bladder. Pressures were then applied to the urethra as they were to the bladder.

Results. I. *Effect on blood pressure and nictitating membrane of distending and emptying the bladder.* In all experiments there was a rise in b.p. upon distention of the bladder. The usual latent period of this reflex was 2 to 7 seconds (fig. 1a). This rise was abrupt and the b.p. remained at peak level for 30 seconds to a minute and then fell very slowly to a lower level with continued distention. On sudden emptying of the distended bladder the b.p. was seen to fall fairly rapidly to its original level, rarely with a negative rebound. In about one-third of the cases five or six extra systoles occurred during the distention of the bladder. On emptying they were somewhat more frequent.

The effects on the n.m. of increased bladder pressure were variable. Reflexes were elicited in the majority of the male cats, but only rarely in the females. The sex difference in reflex responses of the n.m. was not due to stimulation of the urethra, for distention of it alone produced no reflexes. Other variables were the time relations and direction of responses. Most frequently the n.m. relaxed on bladder distention coincidentally with the b.p. rise (fig. 1a, b). At other times, however, the n.m. contracted either simultaneously with the rise in b.p. or several seconds later, and then returned gradually to its original level upon emptying the bladder (fig. 1c). In those instances in which the n.m. was inhibited there followed a contraction with release of bladder pressure after a latent period averaging

5 to 10 seconds, although at times this contraction was almost immediate or as late as 30 seconds (fig. 1a, b).

Respirations were recorded in some experiments, and the rate was found to decrease when the n.m. relaxed and to increase when it was unaffected or contracted (fig. 1b, c).

Cutting the vagi and depressor nerves, denervating the carotid sinuses, and tying the adrenals did not alter these reflex responses. B.p. responses were identical under the anesthetics used, but the n.m. responded only with light urethane anesthesia.

II. *Quantitative relations between bladder pressure, bladder volume and blood pressure.* Measurements were made on the relation of different

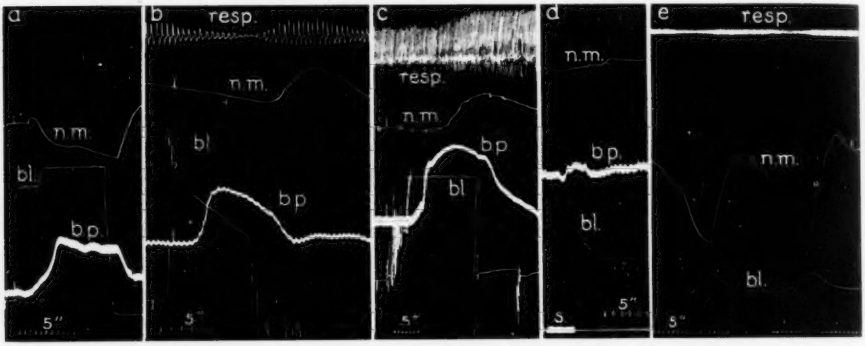


Fig. 1. Cats under urethane anesthesia. *bl.*, bladder pressure. *b.p.*, blood pressure; *n.m.*, isotonic record of nictitating membrane; *resp.*, respiration; *s.*, signal. Time, in 5 second intervals, at bottom of charts. a, bladder pressure raised 9.2 cm. Hg by reservoir; b, 80 cc. saline introduced into bladder in 10 seconds by syringe; c, same as b; 40 cc. saline; d, electrical stimulation of peripheral ends of cut pelvic nerves at *s*. Harvard inductorium, 6 cm.; 3 volts in primary; e, spontaneous activity.

bladder pressures and volumes, taken in random order, upon the rise in b.p. In all instances the vagi were cut and the carotid sinuses denervated. Control readings were included with the bladder displaced extra-abdominally. A definitely consistent relationship existed between bladder pressure and b.p. as shown by the smooth curve in figure 3A. There was a fairly rapid rise at first with the asymptotic portion at bladder pressures in the maximal range of normal. The smallest effective pressure in six experiments was about 1 cm. Hg, and the maximal effect from pressures in the vicinity of 10 cm. Hg. There was, however, marked scattering of points in the graph of bladder volume and b.p., particularly when the volume exceeded 80 cc. (fig. 3B).

III. *Effect of cutting the hypogastric and pelvic nerves and of stimulation*

of the pelvics. In four cats both hypogastric nerves were tied and cut just distal to the inferior mesenteric ganglia. This did not alter the character or magnitude of the b.p. response to distention of the bladder. The reflexes were also obtained after cutting only the pelvics. After both hypogastrics and pelvic nerves were severed the blood-pressure response was abolished. These results are in contrast to those of Talaat (13) who states that "cutting the pelvic visceral nerves has no effect on this pressor response while cutting the hypogastric nerves completely abolished it in 3 preparations and greatly reduced it in two others."

Stimulation of the peripheral ends of the cut pelvic nerves, with hypogastrics intact, produced definite contraction of the bladder and rise in bladder pressure as high as 2.4 cm. Hg with a slight rise in b.p. (fig. 1d) and in one instance a contraction of the n.m.

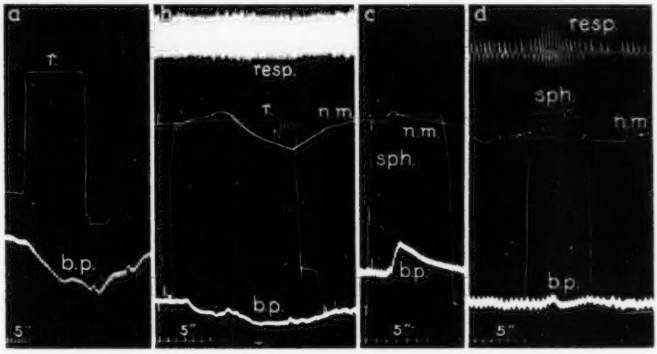


Fig. 2. Same as figure 1. *r.*, dilatation of rectum; *sph.*, dilatation of sphincter ani. For description see text.

IV. *Spontaneous movements of the bladder and nictitating membrane.* In several experiments the bladder contracted spontaneously. On one occasion recordings were also made of respirations and of movements of the n.m. Rhythmical diminution of respiratory rate, relaxation of n.m. and bladder contraction occurred nearly simultaneously about every 110 seconds (fig. 1e).

V. *Effect on blood pressure and nictitating membrane of dilating the rectum.* Coincident with dilatation of the rectum the b.p. consistently fell (fig. 2a, b). This was a slight, sustained, slow fall accompanied in some instances by a relaxation of the n.m. (fig. 2b). Dilatation of the anal sphincter, however, evoked opposite effects, i.e., a sharp rise followed by a fairly sharp fall in b.p. and contraction of the n.m. (fig. 2c). Respirations either remained unchanged (fig. 2b) or increased in rate and depth on sphincter dilatation especially in animals under light anesthesia (fig. 2d).

Discussion. It appears certain that the stimulus for the reflexes obtained arises from sensory end-organs in the bladder for the following reasons. In the first place, the bladder was stripped of its peritoneum and distended extra-abdominally without change in results (p. 33). Secondly, trauma to the exposed tissues cannot be implicated because manipulation of the free peritoneal edges produces prompt contractions of the n.m. which does not occur upon bladder distention. Thirdly, it has been shown that urethral distention does not elicit reflex changes in b.p. or in the n.m. (p. 33). Finally, the stimulus for the reflexes does not arise in the ureters, because the ureters are not distended by the bladder pressures employed, and, furthermore, because the reflexes are obtained after section of the ureteral afferent nerves, as well as upon stimulation of the pelvic nerves peripherally (fig. 1d).

Rosenblueth and Schwartz (loc. cit.) previously have shown the independent action of the n.m. and b.p. in reflex response to stimulation of mixed afferent nerves, and Morison and Rioch (8) have made similar observations on stimulation of the forebrain. This lack of parallelism in response of b.p. and n.m. to a physiological stimulus is also illustrated in the present study, for the most frequent reaction obtained upon distention of the bladder is prompt inhibition of the n.m. with simultaneous rise of b.p. Respiration, however, tends to respond in the same sense as the n.m.

The n.m. responds by both contraction and relaxation in the same experiment (fig. 1a), suggesting the simultaneous activation of both excitatory and inhibitory afferents. The late contraction after release of bladder pressure should probably be regarded as a positive rebound since it follows inhibition. This is unlike the usual rebound where contraction is practically instantaneous on cessation of the stimulus. The secretion of adrenalin is not the cause of this late rise, for it is obtained after both adrenal glands are tied. The phenomenon may be explained by assuming that after-discharge of excitation outlasts that of inhibition, although the inhibition is more effective while present. It seems likely that higher centers influence the after-discharge, for the degree and type of anesthesia affect the responses of the n.m. but not those of the b.p. which follow the course of the stimulus more closely. Further, except for the difference in time relations, a close parallel exists with the rebound contraction of the n.m. following stimulation of the sciatic nerve, reported by Morison and Rioch (loc. cit.) and shown to be dependent on the presence of the frontal lobes.[1]

[1] Further analogy with the results of these authors was present in two experiments. In one the procedure of opening the skull abolished the responses of the n.m. In the other, ablation of both frontal lobes had this effect, though the reflex was still present after only the right frontal lobe had been amputated.

In the experiment in which spontaneous contractions of the bladder and n.m. and change in respiratory rate were recorded the time relations indicate that some higher center discharges rhythmically, causing these three effects simultaneously. In this instance, as contrasted to the experiments quoted in the preceding paragraph, all responses are in the same direction (fig. 1e), i.e., excitation of the sympathetic and respiration with inhibition of the parasympathetic and vice versa.

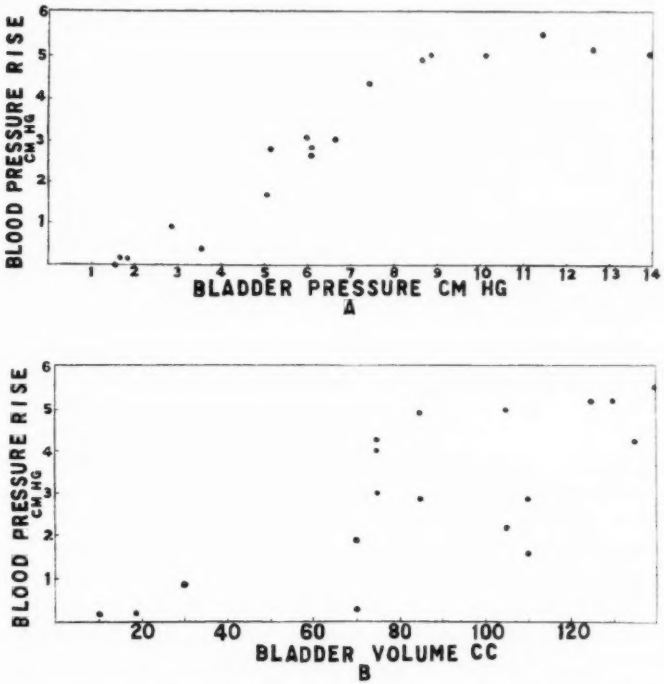


Fig. 3. Quantitative relationship A, between bladder and blood pressure response; and B, between bladder volume and blood pressure response. For discussion see text.

The studies on the bladder volume, bladder pressure, and rise in b.p. show a quantitative relationship between bladder pressure and b.p. response (fig. 3A) which is not true of bladder volume (fig. 3B). This indicates that the sensory end-organs of the bladder respond to pressure, not to distention. What relationship there is between b.p. and bladder volume is apparently secondary to the relationship of bladder volume and pressure.

The rate of adaptation of the sensory end-organs stimulated in these experiments on the bladder and rectum is slow, as indicated by the pro-

longed b.p. responses. This is further borne out by the work of Talaat (loc. cit.) who recorded the afferent discharges from the pelvic nerves by means of the oscillograph. Stretching the sphincter, on the contrary, evokes only momentary rises in b.p. indicating end-organs with rapid adaptation.

It may be objected that some of the pressures applied in these experiments and the rapidity of filling are not consistent with normal physiological conditions. However, the maximal normal tension in the bladder is reported to be between 80 and 130 cm. of water (5), and the great majority of pressures used were within these limits. Moreover, the rate of filling was no more rapid than the increase of pressure resulting from normal bladder contraction, and it has been shown that such contraction, produced by stimulation of the pelvic nerves, evokes these reflexes (fig. 1d).

The subjective sensory findings in patients upon vesicle distention have been reported by numerous authors (2, 6, etc.), but possible concomitant reflex changes in b.p., respirations, etc., have not been investigated in man. Until this is done and further data are available concerning other visceral afferents, generalizations as to the functional significance of these reactions are not justified.

I should like to express my thanks to Dr. D. McK. Rioch under whose guidance and in whose laboratory these experiments were carried out.

SUMMARY

In cats under urethane anesthesia the reflex responses of the nictitating membrane and blood pressure to distention of the bladder and rectum have been recorded. The blood pressure rises consistently (fig. 1a, b, c), and a quantitative relationship to bladder pressure has been found (fig. 3A). The nictitating membrane occasionally contracts (fig. 1c), but usually relaxes and, in the latter cases, shows a positive rebound when the bladder is emptied (fig. 1a, b). The afferent pathway includes both the hypogastric and pelvic nerves. Dilatation of the rectum produces inhibitory effects on the blood pressure and nictitating membrane (fig. 2b), while dilatation of the sphincter produces excitation (fig. 2c). The sensory end-organs involved in the bladder respond to pressure and not to distention. They show slow adaptation as do those in the rectum, whereas the sphincter end-organs adapt rapidly.

REFERENCES

(1) Acheson, G. H., A. Rosenblueth and P. F. Partington. This Journal **115:** 308, 1936.
(2) Barrington, F. J. F. Brain **56:** 149, 1933.
(3) Campbell, M. F. J. Urol. **17:** 371, 1927.
(4) Carlson, A. J. and A. B. Luckhart. This Journal **55:** 31, 1921.

(5) Creevy, C. D. Arch. Surg. **29**: 723, 1934.
(6) Denny-Brown, D. and E. G. Robertson. Brain **56**: 397, 1933.
(7) Morin, G. and J. Vial. C. R. Soc. Biol. **115**: 1122, 1934.
(8) Morison, R. S. and D. McK. Rioch. This Journal (to be published).
(9) Owen, S. E. Am. Heart J. **8**: 496, 1933.
(10) Rosenblueth, A. and H. G. Schwartz. This Journal **112**: 422, 1935.
(11) Scott, H. G. and A. C. Ivy. Arch. Int. Med. **49**: 227, 1932.
(12) Sherrington, C. S. Med. Chir. Trans. **82**: 449, 1899.
(13) Talaat, M. J. Physiol. **89**: 1, 1937.

TIME RELATIONS OF RESPONSE TO REPEATED WATER INGESTION

H. L. WHITE

From the Department of Physiology, Washington University School of Medicine, Saint Louis

Received for publication July 6, 1937

If the view of Klisiecki, Pickford, Rothschild and Verney (1933) is correct, that the lag of water output behind absorption represents the time required for a sufficient fall in the level of postpituitary antidiuretic principle, it would be predicted that if a second drink is given during the diuresis of a preceding drink the delay between the second drink and the onset (and time to maximum) of the corresponding diuresis should be less than with the first drink. If, on the other hand, the lag represents the time required for the tubular cells to reach a new and higher degree of hydration it might also be expected that the second lag would be shorter than the first; such behavior would be compatible with but not demanded by the "tubular hydration" hypothesis, while it is demanded by the "hypopitressinemia" hypothesis.

Klisiecki, Pickford, Rothschild and Verney say, "We have, however, on several occasions given a second dose of water to a dog before the diuresis from the first has subsided, and have observed the earlier onset which theory demands". Conversely, an increased delay was observed with dehydrated animals, which they explain as due to the longer time (1) for the water load to reach the level requisite for inhibition of posterior lobe secretion and (2) for the higher concentration of antidiuretic principle in blood and kidneys to fall to the level at which water is released. No data are given.

Newton and Smirk (1934) find that the delay in diuresis behind water load in acutely decerebrated and hypophysectomized cats is just as great for the second and third water dose as for the first. But they waited until urine flow had returned to normal between doses, and preceding water load had been eliminated. The conditions of their experiments do not permit a testing of the concepts of the first paragraph; they were not designed for that purpose.

This paper reports on experiments on normal men in which the delay in water output in response to a second dose of water given soon after the crest of the diuresis of a preceding drink was compared with the delay after

the first drink. Frequent blood samples were taken; on these serums electrical conductivity and, in some cases, viscosity were followed as an index of plasma dilution. Six such experiments have been done on three subjects.

METHODS. The subjects took nothing by mouth after 10:00 p.m. the evening preceding the experiment except 250 cc. of tap water on arising at 7:30 a.m. Beginning about 10:00 a.m. the subject emptied his bladder every 15 minutes until 4 to 8 control outputs were obtained. He then drank 1 to 1.5 liter of tap water within 5 to 7 minutes. Collections every 15 minutes were continued and some time after the crest of diuresis, while urine flow was still above normal or a significant water load persisted, a second identical drink was taken, collections continuing until output was well back toward normal. From 8 to 10 blood samples were taken, with a minimum or no stasis, from an antecubital vein at the level of the xiphoid. The subjects sat throughout the experiments except while voiding. Room temperature was 24 to 26°C.; at no time was there any sensible perspiration. Serum conductivity and viscosity were determined as previously described (White and Findley, 1937); a change of 0.0001 in specific conductivity indicates a change of 5.5 mgm. per cent NaCl and a change of 0.1 in relative viscosity a change of 0.72 gram per cent protein.

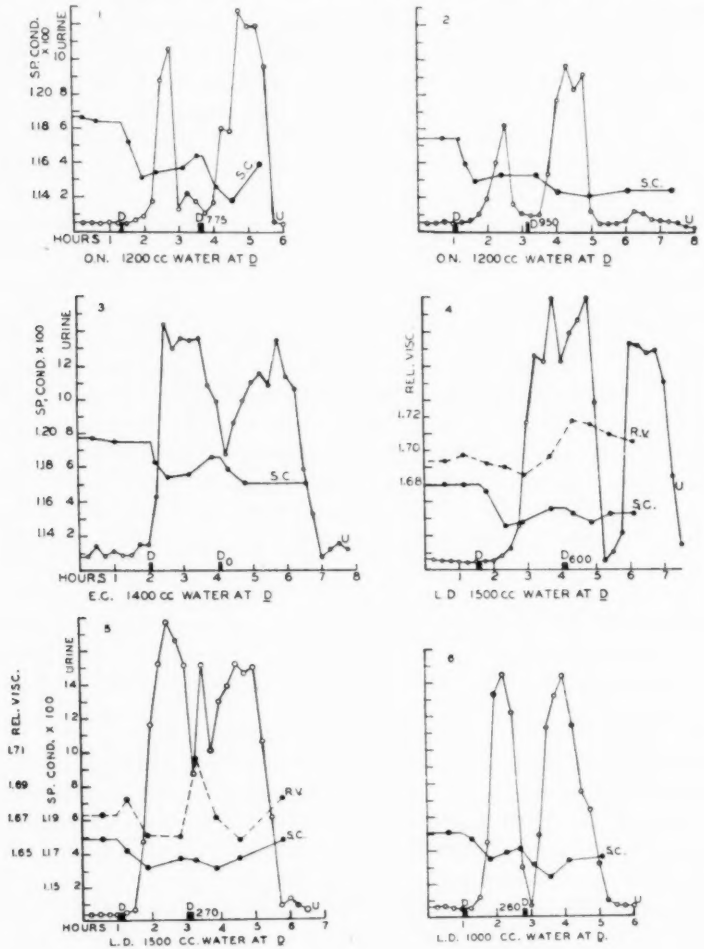
RESULTS. The results are seen in figures 1 to 6. Earlier findings of a drop in serum electrolyte preceding the diuresis are confirmed. The behavior of the serum protein (figs. 4 and 5), as evidenced by viscosity, is somewhat irregular. The high value following the second drink in figure 5 is probably erroneous; very slight stasis suffices to raise serum protein significantly. The protein changes are obviously too small to be causally related to the changes in water output. The conductivity curves show that the course of serum dilution after the second drink does not differ from that of the first.

The average of the 4 experiments 1, 2, 5 and 6 shows an interval of 83 minutes from beginning of first drink to crest of diuresis and a corresponding interval of 83 minutes for the second drink; the beginning of the rise was possibly somewhat sooner with the second drink than with the first.

The course of the urine output after the second drink in experiment 4 is such that one cannot assign a value to the time to maximum; an initial rise was followed by a marked fall, after which came a second rise. This fall was almost certainly due to a vasomotor disturbance; it is seen not to be due to any disturbance of blood dilution. Glomerular filtration probably fell abruptly. This seems to us the most probable explanation, although the subject said he felt no symptoms of faintness. The same subject in experiment 6 showed a normal course; in experiment 5 there is some irregularity.

Experiment 3 is considered separately because of the unprecedented lack

of any lag between water absorption and increase in urine output with the first drink. This cannot be ascribed to a chronic lack of pitressin, since this subject's 24 hour urine output is normal. It has not been possible to



Figs. 1–6. Response of serum specific conductivity and relative viscosity and of urine flow to two drinks of water. Urine flow in cubic centimeter per minute.

have this subject return for further study. He also showed the unexpected finding of a much longer delay in response to the second drink than to the first.

A complication of some of the experiments is that by the time the crest of the first diuresis had been passed a considerable part of the ingested water had been eliminated. While extrarenal water loss under the conditions of these experiments is slightly greater than metabolic water production, no significant error in estimating the water load at the time of the second drink is made by considering only ingested water and urine output. The water load at the time of the second drink, i.e., the difference between the amount ingested at the first drink and the urine output between first and second drinks, is shown in each figure near the black rectangle indicating the second drink. In experiment 3 there was no water load at the time of the second drink but urine flow was well above normal; in the other experiments the load ranged from 260 to 950 cc.

SUMMARY

There is no evidence that a normal human subject develops a lack of pitressin after a large drink of water, where decreased lag of elimination behind absorption of a second drink, ingested while a water load from the first drink persists or while urine flow is well above normal, is taken as evidence of such a lack.

REFERENCES

KLISIECKI, A., M. PICKFORD, P. ROTHSCHILD AND E. B. VERNEY. Proc. Roy. Soc. B **112**: 521, 1933.

NEWTON, W. H. AND F. H. SMIRK. J. Physiol. **81**: 172, 1934.

WHITE, H. L. AND T. FINDLEY, JR. This Journal **119**: 740, 1937.

SOME EFFECTS OF ZINC AND IRON SALTS ON THE HYPOGLYCEMIC ACTION OF INSULIN IN RATS

N. R. BLATHERWICK, MARY E. EWING AND PHOEBE J. BRADSHAW

From the Biochemical Laboratory, Metropolitan Life Insurance Company, New York

Received for publication July 8, 1937

It was first shown by Maxwell and Bischoff (1) that the addition of partially neutralized ferric chloride to insulin caused an augmentation of the physiological response as measured by blood sugar changes in both rabbits and rats. Four milligrams of ferric chloride and 2 units of insulin per cubic centimeter and per kilogram were given intramuscularly. Scott and Fisher (2) gave their findings with various salts of zinc showing that this metal caused a prolongation of the hypoglycemia in rabbits and decreased the incidence of convulsions in mice. The solutions used in the rabbit experiments contained 0.1 per cent zinc while those used in the mice experiments contained about 0.01 per cent zinc. In additional experiments, they found that nickel chloride had an action similar to that of zinc while cobaltous chloride and potassium alum had little effect on the activity of insulin. Scott and Fisher (3) found that the addition of 1.0 mgm. of zinc to 500 units of insulin had only a slight effect in prolonging the hypoglycemia in rabbits but that this small quantity of zinc when added to protamine-insulin mixtures caused a significant prolongation of the insulin action. Fazekas and Himwich (4) reported that when from 6.5 to 13.0 mgm. of zinc as zinc sulfate and 10 units of insulin were injected into dogs no hypoglycemia was observed over a period of 6 hours. In experiments on cats in which the zinc was reduced to 1.3 mgm. and the insulin was increased to 2 units per kilogram hypoglycemia resulted. In dosage of 11.2 mgm., aluminum as aluminum chloride abolished hypoglycemia but when the quantity was reduced to 5.6 mgm. and given with 10 units of insulin lowering of the blood sugar occurred. They also observed that 10 mgm. calcium as calcium chloride and 10 mgm. magnesium as magnesium sulfate exerted no inhibitory effect on insulin action. Bischoff and Jemtegaard (5) found that 0.1 mgm. copper per unit and 0.1 mgm. aluminum per unit prolonged the hypoglycemia in rabbits.

This review of the literature indicates that the effects on hypoglycemia produced by the addition of metals to insulin are subject to considerable variations and that the latter are probably dependent upon factors such

as the concentration of the metal, whether or not the metal is in solution, and the ratio of metal to insulin. The data reported in this paper deal with these points. Because of the current interest in insulin products with retarded action, we have also studied the hypoglycemic action of crystalline insulin.[1]

Rats of both sexes were fasted 24 hours before the subcutaneous administration of insulin. Blood sugar was determined by the method of Folin and Malmros on samples obtained from the tail at the following intervals: before the injection of insulin, 1½ hours, 3 hours, 5 hours, and 6½ hours after insulin was given. Dilution of the regular U 40 insulin was made with water.

The results with regular insulin presented first in table 1 show that when 0.5 unit per 100 grams body weight was given the greatest hypoglycemia occurred at 1½ hours with a return to an apparent level somewhat lower than the initial at 5 hours.

There seemed to be a significant difference at the 5 hour period when Sahyun's crystalline insulin was given. In this case the blood sugar did not return to the control value until 6½ hours after the injection. The markedly prolonged action of crystalline insulin observed in diabetic patients did not occur in normal rats.

The injection of a mixture containing 4 mgm. of neutralized ferric chloride and 4 units of insulin per cubic centimeter resulted in an augmentation and prolongation of insulin activity with a shifting of the point of greatest hypoglycemia from the 1½ to the 3 hour interval. This result is similar to that of Maxwell and Bischoff (1) when twice as much ferric chloride in relation to insulin was given.

A mixture containing 5 mgm. of neutralized zinc chloride and 4 units of insulin per cubic centimeter caused a marked augmentation and prolongation of insulin action. The point of maximum hypoglycemia was shifted from the 1½ to the 5 hour interval with respective values of 34.7 and 28.1 mgm. per 100 cc. This result is to be contrasted with that obtained when a solution containing 5 mgm. of zinc chloride (not neutralized) and 4 units of insulin per cubic centimeter were given. In this case, hypoglycemia did not occur until the 5 hour period with a further lowering in blood sugar at 6½ hours. It is probable that the rather concentrated solution of zinc chloride caused tissue injury at the site of the injection thus preventing absorption of insulin. The next three experiments were planned to find the optimum concentration of zinc chloride (not neutralized) which would insure prolongation of insulin action. It is seen that a solution containing 0.8 mgm. of zinc chloride and 4 units of insulin per cubic centimeter gave better results than did one

[1] The crystalline insulin, as well as the amorphous insulin, was generously supplied by Dr. Melville Sahyun of Frederick Stearns and Company.

TABLE 1

Effects of iron and zinc salts on insulin hypoglycemia

NUMBER OF RATS		INSULIN		METALS		BLOOD SUGAR					REMARKS
Male	Female	Unit per 100 grams	Units per cubic centimeter	$ZnCl_2$	$FeCl_3$	Initial	1½ hours	3 hours	5 hours	6½ hours	
				mgm. per cc.	*mgm. per cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	
6	6	0.5	4.0			80.4±2.6	34.7±1.2	47.1±3.5	76.8±1.5	76.0± 1.9	Amorphous insulin
8	3	0.5	4.0, 1.6			81.0±3.3	37.9±1.2	44.9±3.3	71.0±1.7	77.2± 0.9	Sahyun's crystalline insulin
6	6	0.5	4.0		4.0	79.0±1.2	38.4±1.3	31.3±1.2	37.1±2.4	46.8± 3.5	NaOH was added to the iron solution until neutral to litmus
3	3	0.5	4.0	5.0		75.5±5.6	44.2±3.3	33.1±0.3	28.1±1.1	29.1± 2.3	NaOH was added to the zinc solution to approximate neutrality
6		0.5	4.0	5.0		77.4±2.1	78.8±1.9	76.1±1.9	70.1±4.7	56.0± 6.2	Natural reaction of the zinc solution
3	3	0.5	4.0	0.8		82.1±2.1	51.9±6.0	36.7±3.3	33.7±3.0	41.1± 5.8	Natural reaction of the zinc solution
	6	0.5	4.0	0.7		83.6±2.2	48.5±2.0	38.6±1.6	50.7±9.1	60.7±11.1	Natural reaction of the zinc solution
	6	0.5	4.0	0.6		79.1±1.0	42.9±4.4	36.4±2.9	49.2±5.8	65.1± 1.5	Natural reaction of the zinc solution
	6	0.5	4.0	0.5	0.5	82.3±2.4	41.8±2.0	33.6±1.2	41.6±4.6	54.7± 6.2	Natural reaction of the solutions of the metals
	6	0.5	4.0	0.6	0.4	79.1±1.4	46.2±2.7	34.2±4.2	42.2±6.2	51.5± 6.0	Natural reaction of the solutions of the metals
	6	0.5	4.0	0.7	0.3	78.1±2.0	38.9±1.0	33.2±2.0	34.0±2.9	43.2±14.5	Natural reaction of the solutions of the metals
	6	0.4	4.0	0.7	0.3	77.6±1.0	37.9±1.2	34.6±1.4	48.3±2.8	65.4± 2.5	Natural reaction of the solutions of the metals
12		0.5	8.0	0.7	0.3	82.9±2.9	53.5±4.4	38.6±2.7	35.6±3.2	48.4± 4.8	Natural reaction of the solutions of the metals
15		0.7	8.0	0.7	0.3	84.7±1.7	67.1±4.7	51.1±3.9	41.3±2.7	45.7± 4.4	Natural reaction of the solutions of the metals

The mean deviation of the mean was calculated with the formula $\epsilon_M = \sqrt{\frac{\Sigma d^2}{(N-1)\,N}}$

containing 0.7 and another with 0.6 mgm. of zinc chloride per cubic centimeter. The point of maximum hypoglycemia shifted back from the 5 hour to the $3\frac{1}{2}$ hour interval with the two smaller doses of zinc chloride and the final $6\frac{1}{2}$ hour value was decidely higher in both cases. The ratio of zinc to insulin in the optimum solution was roughly 0.1 mgm. of zinc to 1 unit of insulin.

The remaining experiments deal with the effects produced by mixtures of zinc chloride and ferric chloride (both not neutralized) when added to insulin. The results show that a solution containing 0.7 mgm. zinc chloride, 0.3 mgm. ferric chloride, and 4 units of insulin per cubic centimeter was the best. The degree of hypoglycemia produced by this combination appeared to be somewhat greater than that obtained with the solution containing 0.8 mgm. zinc chloride and 4 units of insulin per cubic centimeter; the prolongation of action was about the same in both cases. There was a difference in the character of the blood sugar curve of males and females produced by this combination of metals. The difference was apparently caused by a slower rate of absorption dependent upon the larger dose injected into the male animals. This same effect is clearly apparent in the last experiment in which a slower rate of decrease in blood sugar resulted even though the dose of insulin was increased from 0.5 to 0.7 unit per 100 grams body weight. A number of experiments not recorded in table 1 showed that it is possible to change the character of the blood sugar curve by regulating the size of the dose injected, the proportions of the metals and the dose of insulin remaining constant. Our attention was called to this phenomenon when we found that large male rats responded with a slower rate of decrease of blood sugar than did the smaller ones. It was first thought that this delayed action was perhaps dependent upon a possible interference of subcutaneous fat with the absorption of insulin. But other experiments showed that this delayed absorption could be produced in the smaller animals by making the dose contain the same amount of metals as was given the larger ones. For this reason, it is preferable in such studies to use only female rats which do not vary so greatly in weight as do the males.

Bischoff (6) has made the following statement about the various insoluble insulin combinations: "The marked decrease in activity observed when the histone combination is given intramuscularly and assayed in the conventional manner is markedly in contrast to the results obtained with other insoluble combinations such as the tannate, iron and zinc combinations. The two former produce an augmentation in effect, the latter a prolongation of effect with a more shallow hypoglycemic curve." Our experiments do not confirm this belief of Bischoff concerning the effects of zinc, since they showed both an augmented and a prolonged effect. An experiment demonstrating the augmentating effect of neutral-

ized zinc chloride follows. Three female rats were given a dose of 0.2 unit per 100 gram weight of a mixture containing 2 units of insulin and 2.5 mgm. zinc chloride per cubic centimeter. The average blood sugar values obtained were 71.7, 31.1, 29.9, 43.9 and 67.3 mgm. per 100 cc. for the regular time intervals. These results indicate that the zinc had increased the hypoglycemic action at least twice.

Our experiments deal only with such quantities of the metals as give the maximum effects on the hypoglycemic action of insulin. They do not have an immediate bearing upon any possible effects produced by the much smaller quantities present in amorphous and crystalline insulin; neither do they apply to the demonstrated effect of very small quantities of zinc added to mixtures of protamine and insulin.

SUMMARY

Investigation on rats of the effects on hypoglycemia produced by adding salts of zinc and iron to solutions of insulin showed that these are dependent upon factors such as the concentration of the metal, whether or not the metal is in solution, and the ratio of metal to insulin.

REFERENCES

(1) Maxwell, L. C. and F. Bischoff. This Journal **112:** 172, 1935.
(2) Scott, D. A. and A. M. Fisher. J. Pharmacol. Exper. Therap. **55:** 206, 1935.
(3) Scott, D. A. and A. M. Fisher. J. Pharmacol. Exper. Therap. **58:** 78, 1936.
(4) Fazekas, J. F. and H. E. Himwich. J. Pharmacol. Exper. Therap. **58:** 260, 1936.
(5) Bischoff, F. and L. M. Jemtegaard. This Journal **119:** 149, 1937.
(6) Bischoff, F. This Journal **117:** 182, 1936.

THE INFLUENCE OF THE CEREBRAL CORTEX ON PERIPHERAL CIRCULATION

J. O. PINKSTON AND D. McK. RIOCH

From the Laboratories of Physiology and the Department of Anatomy in the Harvard Medical School and the Department of Pharmacology in the Vanderbilt University School of Medicine

Received for publication July 8, 1937

The effects of ablation of various cortical areas upon the peripheral circulation have been considered by a number of authors, and the pertinent literature has been reviewed in detail recently by Kennard (1935, 1936) and Bucy (1935). In general it may be said that lesions involving areas 4 and 6 (Brodmann) result in durable changes in the cutaneous vasomotor reactions of the contralateral half of the body. A discrepancy exists, however, in the type of vasomotor change reported in the monkey and that in the dog. In the former, after experimental ablation of area 4 or 6, Kennard (1935) found a chronic vasoconstriction, with delayed dilatation on exposure to heat. The effects were more pronounced after area 6 lesions than after those of area 4. Pinkston, Bard and Rioch (1934), working on the dog, reported a continued vasodilatation with decrease of reflex vasoconstriction on exposure to cold, following removal of the sensory-motor area. The present series of experiments were therefore performed in order to investigate further this difference, as well as the cortical localization of the phenomena.

PROCEDURE. Briefly stated the procedure consisted in determining the skin temperature of symmetrical points on the extremities of the two sides before and after ablation of different cortical areas in monkeys. As it was desirable to employ animals amenable to handling, the studies were carried out on four fairly tame spider monkeys and three sooty mangabeys.

The cortical ablations were performed with aseptic precautions, under dial anesthesia (Ciba's sterile solution, 0.4 to 0.8 cc. per kilogram body weight, intraperitoneally). The skull was opened with trephine and rongeurs; the cortical area to be ablated was outlined with a scalpel and removed with a sharp spatula. The wound was closed in three layers—dura, galea and skin. In four of the animals, after completion of the temperature studies following the first cortical lesion, a second lesion was made on the opposite side.

The motility, posture, etc., of the animals were observed throughout the post-operative course for possible correlation with the other data.

Temperature readings, in degrees Fahrenheit, were obtained by means of thermocouples and a Leeds and Northrup temperature recorder (Micromax) from two symmetrical points on the skin (soles or palms), from the rectum, and from the room air at intervals of four minutes for observation periods of twenty to forty minutes. The average of the several determinations during a given period was taken as the temperature record at that time. The skin thermocouples were applied by special holders in the form of metal shields which could be fastened very snugly to the soles or palms with strips of adhesive tape.

As a general rule, during a series of temperature readings, the monkeys were placed in a holding box which prevented gross movement without causing undue excitement in the animal. Movable partitions in the box allowed for adjustment to monkeys of various sizes. Particular care was exercised to prevent any constriction of the limbs such as would affect the circulation.

At the conclusion of the studies the portions of the brains involved were examined histologically (thionine stain) and graphic reconstructions of the lesions and adjoining areas were made.

Results. The surface temperature determinations before cortical ablation showed differences on the two sides irregularly and seldom of more than a fraction of a degree Fahrenheit, except in the case of monkey 4 (see below). In all cases after lesions involving areas 4 or 6, however, the temperature of the contralateral extremity was found to be lower than that of the ipsilateral. The extent of the difference between the two varied considerably on successive days and in some cases tended to decrease somewhat with the passage of time, but in no instance was the normal relationship reëstablished during the period of observation (up to three months). Lesions involving area 4 resulted in more pronounced differences (maximum 8.4°F., average of all determinations 3.17°F.) than did those involving area 6 (maximum 5.3°F., average of all determinations 2.04°F.). The following two experiments are illustrative.

Monkey 6. Sooty mangabey, male. This was a quiet animal which accepted the experimental procedure with little or no resistance. After a preliminary period of observation, area 4 on the left was ablated on July 14, 1936 (see fig. 1, A). Recovery from the operation was uneventful and the animal showed the usual symptoms of motor paralysis on the right throughout the period of observation which ended September 4, 1936. Repeated temperature determinations were made during this time and the differences between the two sides are recorded in figure 2, A. Direct observation of sweating of the face, palms and soles failed to reveal any consistent changes.

Monkey 5. Sooty mangabey, male. This was a quiet animal and easy to handle. It was studied from June 30 to September 4, 1936. On July 7

a lesion was made in the right cortex, involving mainly area 6 (see fig. 1, B). Recovery was uneventful and paralytic symptoms lasted for three days. On July 21 a second operation was performed, with removal of area 4 on the left side (fig. 1, C). The resulting motor paralysis remained, but no constant differences in the sweating responses were observed during the period of observation. The differences in the skin temperature on the two sides are shown in figure 2, B.

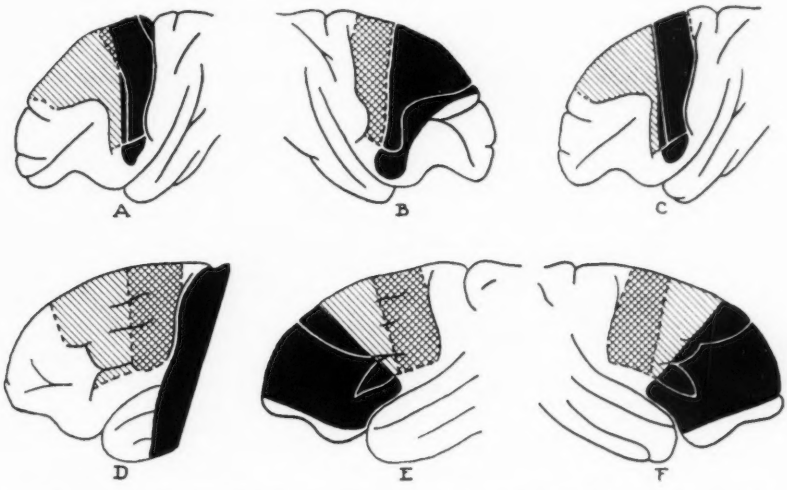


Fig. 1. Semidiagrammatic representation of the rostral halves of the operated cerebral hemispheres of the monkeys described in the text. The hemispheres are shown as viewed from in front and above, so that a perpendicular to the paper would make an angle of about forty-five degrees with each of the three planes of reference.

A. Left hemisphere, monkey 6. *B* and *C*. Right and left hemispheres, monkey 5. *D*. Left hemisphere, monkey 4. *E* and *F*. Left and right hemispheres, monkey 1.

Area 4 is represented by cross-hatching; area 6, by parallel lines. Extent of cortical ablation is indicated by solid black.

In figure *D*, only the rostral margin of the ablation is shown, all the neocortex caudal to this level having been removed.

Several experiments afforded evidence that the cortical influence on the peripheral circulation is localized principally, if not entirely, to areas 4 and 6. In one experiment, monkey 4, all of the neocortex posterior to the central fissure, with the exception of that over the tip of the temporal pole, was removed September 13, 1935 (see fig. 1, D). This animal was a large, rather vicious male spider monkey and struggled considerably when restrained, so that only a few temperature determinations which could be considered reliable were obtained. These showed marked ir-

regularities in the pre-operative period, but no significant changes following the cortical ablation.

In three experiments on two monkeys the lesions involved the greater portion of the frontal lobe, including one-half to two-thirds of area 6. The temperature changes were similar to those reported above for the area 6 lesions. In monkey 1, a quiet, female spider monkey, portions of the cortex were ablated on the left side April 23 and on the right August 7, 1935 (see fig. 1, E and F). Transient motor paralysis followed in each case. The right side remained cooler than the left after the first operation (4/26, 1.79°; 6/27, 1.13°; 7/23, 5.0°; 8/5, 4.7°). There was no reversal of laterality following the second operation as occurred in monkey 5 (see above), though a considerable decrease in the previous asymmetry was apparent (8/8, 1.1°; 8/16, 2.5°; 8/22, 1.3°).

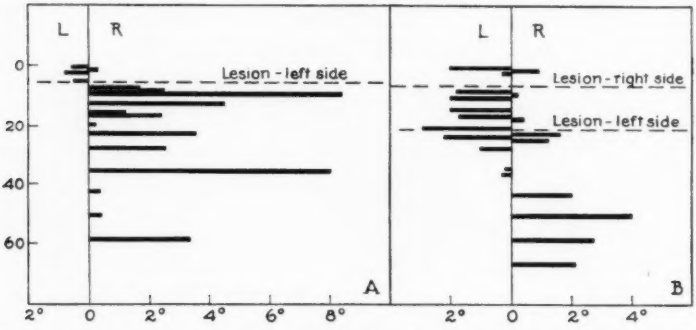


Fig. 2. Differences in temperature between the two sides of the body before and after the cortical ablations shown in figure 1, *A*, *B* and *C*.

A. Monkey 6. B. Monkey 5. The length of the horizontal lines represents the relative coldness in degrees Fahrenheit of the left and right sides respectively. Time is represented vertically in days.

A number of factors which influence skin temperature were investigated, such as body temperature, the temperature and relative humidity of the room air, sweating, muscular activity and paralysis. These will not be reported in detail since, except in the case of struggling, no consistent relationships could be ascertained between them and the difference of the skin temperature on the two sides after ablation of areas 4 or 6. Struggling frequently caused marked fluctuation. In cases of vigorous and prolonged struggling, the skin temperature almost invariably rose, the final level usually being equal on the two sides even in the operated animals. Sweating, so far as could be noted by direct observation, was very irregular. At times it was more marked on the affected and at other times on the normal side. Indeed, the face and hand on one side and the foot on the other occasionally showed the greater moisture. With regard to the

influence of motor paralysis on the surface temperature, it should be noted that the temperature differences outlasted the transient paralyses in all cases of lesions of area 6 and in one case became more pronounced after the return of function, monkey 1. The relative differences in the degree of paralysis in the animals with area 4 lesions also failed to correspond with the magnitude of the surface temperature changes.

Discussion. The assumption that the post-operative inequality of skin temperature reported above was due to unequal activity of the vasomotor system on the two sides is justified in view of the absence of correlation with other possible factors, as pointed out in the preceding paragraph. Mention may also be made that the danger of constriction of the extremities during temperature determinations was largely eliminated by the design of the holding box. Any errors, however, which might have been introduced by the technique, not only would have affected the results before as well as after the operations, but also would probably have affected both sides of the body equally.

Early evidence that vasomotor reactions could be evoked by electrical stimulation of the cortex (Eulenberg and Landois, 1876; Howell and Austin, 1899) has since been confirmed by several workers, and, in addition, it has been established that this takes place in the absence of skeletal muscular activity (Dusser de Barenne and Kleinknecht, 1924; Kabat, Magoun and Ranson, 1935; Hoff and Green, 1936; Green and Hoff, 1937). In accord with these findings is the fact that the temperature changes reported here bore no constant relation to the motor symptoms (cf. also Kennard, 1935). It may therefore be concluded that the contralateral, peripheral vasoconstriction which follows lesions of the cortical areas 4 and 6 is due to interference with central mechanisms controlling vasomotor reactions. The mode of control, the efferent paths, as well as the possible subcortical centers involved, however, remain obscure.

In general the results of the present experiments confirm those of Kennard (1935). The main discrepancy, namely, the relative importance of area 4 as compared with area 6, may be attributable to differences in technique, since Kennard investigated the reactions of her animals to changes in environmental temperature, whereas, in the present experiments, time was allowed for adjustment to environmental temperature at the beginning of each series of observations. In no case have cortical lesions been found to cause vasodilatation in the monkey (cf. also Kennard, 1936; Hoff and Green, 1936). The opposite effect obtained on dogs (Eulenberg and Landois, 1876; Pinkston, Bard and Rioch, 1934) is therefore probably a species difference. In the absence of comparative studies on other forms it is not possible to say whether this difference is related to encephalization, to peculiarities in temperature control, or to other factors. A number of studies on the human have given conflicting results

(cf. Danielopolu, Radovici and Aslan, 1931; Kennard, Viets and Fulton, 1934; Zollinger and Schnitker, 1934; Bucy, 1935; Kennard, 1936; etc.). The majority of the reported cases showed contralateral vasoconstriction, similar to that in monkeys. A smaller number, particularly some with recent cortical lesions which had occurred suddenly, presented vasodilatations. The cortical localization as shown by Kennard's series of cases (loc. cit.) differed from that in the monkey in that similar results, though somewhat less in degree, were also evoked by lesions in the region of the postcentral gyrus.

SUMMARY

From the palms of the hands or soles of the feet of seven monkeys skin temperature records were taken by means of thermocouples before and at intervals up to three months after operative ablation of certain areas of the cerebral cortex. Lesions involving areas 4 and 6 (Brodmann) resulted in a decrease of the skin temperature of the contralateral side relative to the ipsilateral. This was independent of motor paralysis and persisted throughout the period of observation. Removal of nearly all the neocortex posterior to the central sulcus was without significant effect.

It is concluded that central mechanisms controlling vasomotor reactions are localized in the cortical areas 4 and 6, and that the difference between the monkey and the dog, which shows a vasodilatation (Pinkston, Bard and Rioch, 1934), is a species difference.

REFERENCES

Bucy, P. C. Arch. Neurol. and Psychiat. **33:** 30, 1935.

Danielopolu, D., A. Radovici and A. Aslan. Ztschr. f. d. ges. Neurol. u. Psychiat. **132:** 671, 1931.

Dusser de Barenne, J. G. and F. Kleinknecht. Ztschr. f. Biol. **82:** 13, 1924.

Eulenberg, A. and L. Landois. Virchow's Arch. **68:** 245, 1876.

Green, H. D. and E. C. Hoff. This Journal **118:** 641, 1937.

Hoff, E. C. and H. D. Green. This Journal **117:** 411, 1936.

Howell, W. H. and M. F. Austin. This Journal **3:** xxii, 1899.

Kabat, H., H. W. Magoun and S. W. Ranson. Arch. Neurol. and Psychiat. **34:** 931, 1935.

Kennard, M. A. Arch. Neurol. and Psychiat. **33.** 537, 1935.
Ztschr. f. d. ges. Neurol. u. Psychiat. **155:** 714, 1936.

Kennard, M. A., H. R. Viets and J. F. Fulton. Brain **57:** 69, 1934.

Pinkston, J. O., P. Bard and D. McK. Rioch. This Journal **109:** 515, 1934.

Zollinger, R. and M. T. Schnitker. Science **79:** 540, 1934.

THE CHOLINE OXIDASE OF LIVER[1]

FREDERICK BERNHEIM AND MARY L. C. BERNHEIM

From The Departments of Physiology and Pharmacology and Biochemistry, Duke University School of Medicine, Durham, North Carolina

Received for publication July 10, 1937

In a previous paper we have shown that choline is oxidized by liver suspensions (1). Acetylcholine was not oxidized until it was hydrolyzed, which indicated that the alcohol group on the choline was attacked. The oxidation was carried out at pH 7.2 and the average oxygen uptake was one and a half atoms for every molecule of choline. The fact that choline is oxidized by liver was confirmed by Trowell (2) who used liver slices. We have now studied the oxidation in greater detail and have been able to purify the enzyme and show that it is distinct from the enzyme which oxidizes ethyl alcohol. Evidence from the oxygen uptake and the properties of the end products indicates that in slightly acid solutions choline is oxidized to betaine aldehyde whereas in alkaline solutions it is oxidized to betaine. At intermediate hydrogen ion concentrations it is probable that a mixture of betaine aldehyde and betaine is formed.

EXPERIMENTAL. The liver of a rat is chopped with scissors and ground in a mortar with sand and 0.05 M phosphate buffer pH 7.8. It is pressed through muslin and made up to about 20 cc. with buffer and dialyzed against distilled water for 24 hours in the ice box. It is then centrifuged and the precipitate is suspended in a small amount of water, centrifuged again and finally suspended in twice its volume of phosphate buffer at the desired pH. This preparation contains an active choline oxidase and can be kept for several days in the ice box with little loss of activity. Alone, it takes up very little oxygen and reduces methylene blue very slowly.

The Warburg technique was used for the measurement of the oxygen uptake in these experiments. The choline chloride (Hoffmann-LaRoche) was dissolved in 0.5 cc. of buffer at the required pH and enzyme and buffer were added to make a total volume of 2 cc. in the Warburg vessel. Figure 1 shows the effect of hydrogen ion concentration on the oxidation of choline. At pH 6.7 one atom of oxygen is taken up for every molecule of choline oxidized and at pH 7.8 two atoms of oxygen are taken up. The end points are definite and the curves stop sharply at the theoretical values. On

[1] Part of the expense of this work was defrayed by a grant from the Duke Research Council.

the other hand at pH 7.2 the oxygen uptake stops between 1 and 2 atoms. Figure 2 shows that these results are not due to the different activity of the enzyme but represent different stages in the oxidation. In this case the choline concentration was kept constant and the amount of enzyme was varied and although the rate of the oxygen uptake varies somewhat with the enzyme concentration, the final amount of oxygen which is taken up is dependent only on the pH. Thus 0.5 cc. of enzyme at pH 7.8 carries the

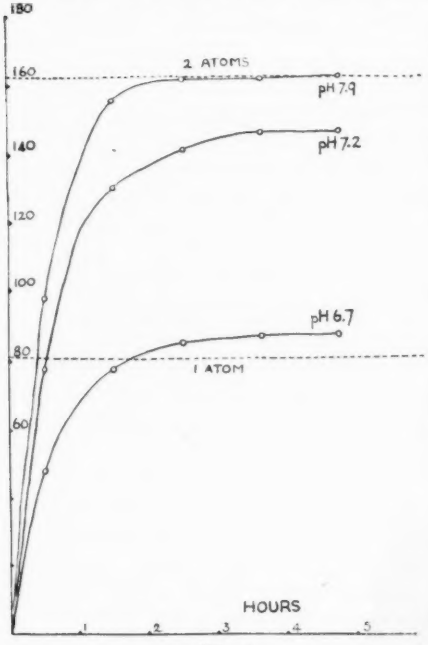


Fig. 1

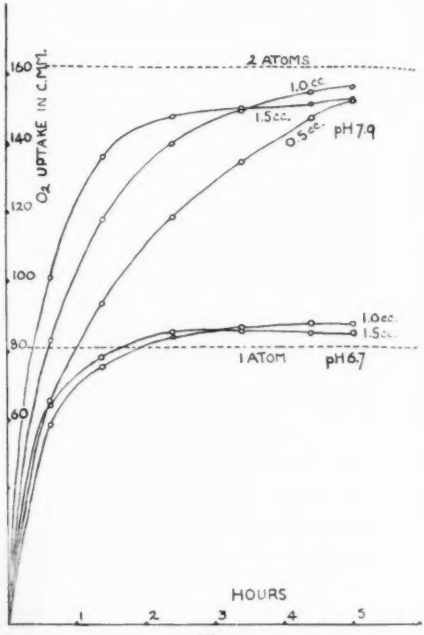


Fig. 2

Fig. 1. The oxygen uptake of 1.0 mgm. choline chloride at various hydrogen ion concentrations.

Fig. 2. The effect of the concentration of enzyme on the oxygen uptake of 1.0 mgm. of choline chloride at pH 6.7 and 7.8.

oxygen uptake to the 2 atom stage whereas 1.5 cc. of the same enzyme solution at pH 6.7 causes an uptake of only 1 atom.

The theoretical values obtained for the oxidation of choline at the two hydrogen ion concentrations suggest that at pH 6.7 choline is quantitatively oxidized to the corresponding aldehyde, namely, betaine aldehyde, and that at pH 7.8 it is quantitatively oxidized to betaine. In order to show this the following experiments were done. From 0.5 mgm. to 1.0 mgm. of choline was oxidized by the enzyme solution at pH 6.7 and 7.8.

When the theoretical oxygen uptake was obtained in each case, 1.0 cc. of 5 per cent trichloracetic acid was added to 2.0 cc. of the enzyme choline mixture. Seven cubic centimeters of water were then added and the precipitate filtered off. An aliquot part of the filtrate was then treated with sodium bisulfite and the estimation of the bisulfite compound was carried out according to the method of Clift and Cook (3). In twelve experiments the yield of bisulfite compound varied from 70 to 90 per cent of the theoretical value when the pH during the oxidation was 6.7 but at pH 7.8 less than 10 per cent of the end product combined with bisulfite. This indicates that at pH 7.8 the amount of aldehyde present at the end of the oxidation was negligible. In one case, when the trichloracetic acid was added before the oxygen uptake at ph 7.8 was complete, 24 per cent of the bisulfite compound was formed which suggests that the oxidation to betaine goes through the aldehyde stage.

Other evidence that an aldehyde was formed at pH 6.7 was obtained as follows. Ten milligrams of choline were oxidized and when the oxidation was complete the protein was precipitated by adjusting to approximately pH 4.5 with acetic acid and immersing for five minutes in a boiling water bath. The filtrate was evaporated to dryness at 40° and the solid extracted with cold absolute alcohol. The insoluble fraction was filtered off and the filtrate again evaporated to dryness. The solid was taken up in water and this solution was highly reducing. It formed a silver mirror in the cold and reduced Fehling's solution. Control experiments with the enzyme solution alone or after the oxidation of the same amount of choline at pH 7.8 were entirely negative.

Attempts to isolate the aldehyde as a semicarbazone or phenylhydrazone failed. Betaine aldehyde was therefore synthesized according to the method of Voet (4). The synthetic aldehyde is highly hygroscopic and comes down as a syrup. A crystalline polymer of the aldehyde is also formed which is readily separated. A solution of the aldehyde gives typical aldehyde reactions, forms a bisulfite compound and a gold or mercury salt. We were, however, unable to obtain a phenylhydrazone or semicarbazone from the synthetic aldehyde although Voet describes the semicarbazone.

The synthetic aldehyde could be oxidized by the enzyme, but only at pH 7.8. Four milligrams of the aldehyde added to the enzyme solution at pH 6.7 caused only a slight oxygen uptake, whereas at pH 7.8 it caused a large uptake. Thus the average uptake at pH 6.7 was 35 cmm. of oxygen and at pH 7.8, 290 cmm. This latter uptake approximately corresponds to the utilization of one atom of oxygen per molecule of aldehyde oxidized.

The evidence for the formation of betaine at pH 7.8 can be summarized as follows. The oxygen uptake per molecule of choline corresponds to the

formation of betaine as does the oxygen uptake of the synthetic aldehyde. The end-product at pH 7.8 shows no reducing properties yet gives a precipitate with mercury and gold salts characteristic of betaine and choline. Like betaine it is insoluble in cold absolute alcohol and in cold glacial acetic acid whereas choline and betaine aldehyde are soluble in these solvents. The evidence for the formation of betaine aldehyde and betaine from choline must be considered presumptive because no cystalline products were obtained but taken as a whole the facts suggest strongly that these substances are formed when choline is oxidized under the given conditions.

The preparation containing the choline oxidase can also oxidize succinic acid, d-proline, tyramine and to a slight extent xanthine. Other substrates such as lactic, acetic, formic, α-glycerophosphoric acids and glucose are not attacked. The succinoxidase, tyramine oxidase and proline oxidase

TABLE 1

The reduction of 1 cc. of 1:5000 methylene blue at pH 6.7 and pH 7.9 by choline in the presence of the enzyme preparation. 37°C. The choline concentration was 10 mgm. per cubic centimeter

BUFFER	pH	ENZYME	CHOLINE	REDUCTION TIME
cc.		*cc.*	*cc.*	*minutes*
1.5	6.7	0.5		105
1.45	6.7	0.5	0.05	35
1.4	6.7	0.5	0.1	28
1.3	6.7	0.5	0.2	20
1.5	7.9	0.5		43
1.45	7.9	0.5	0.05	17
1.4	7.9	0.5	0.1	10
1.3	7.9	0.5	0.2	12

can be obtained in preparations which are unable to oxidize choline. If the choline oxidase in our preparation was to be considered specific, we felt that it was essential to show that it could be separated from the alcohol oxidase of liver, for in both cases the alcohol group is attacked. We found that untreated liver suspension is able to oxidize both substances though the choline oxidase preparation is unable to oxidize alcohol. Conversely, if liver is treated with acetone, a watery extract made from the residue will oxidize alcohol but is unable to attack choline. The nonidentity of these two enzymes is thus proved.

If choline is added to the untreated liver suspension the methylene blue reduction time of the suspension is not appreciably altered. But if the enzyme preparation, which reduces methylene blue very slowly, is used, the addition of choline definitely shortens the reduction time both at pH 6.7 and pH 7.8. This is shown in table 1. The system is thus able

to use methylene blue as well as oxygen as a hydrogen acceptor. Because the synthetic aldehyde in alkaline solutions rapidly reduces methylene blue by itself it was impossible to test the effect of its addition to the enzyme. In acid solution it has little effect in decreasing the reduction time.

KCN in fairly large concentrations definitely inhibits the oxidation of choline. The inhibition is, however, rather short in duration and this accounts for the fact that we originally reported that the oxidation was cyanide insensitive. After half an hour the rate of oxygen uptake with and without cyanide is the same. But under the same conditions and using the same liver suspension the oxidation of alcohol remains almost completely inhibited for two or three hours. Other inhibitors such as fluoride, urethane and physostigmine act on the choline oxidase only in large concentrations. In this respect physostigmine shows none of the specificity that it shows toward the choline esterase.

In the rat, the liver is the most active tissue for the oxidation of choline. The kidney is able to oxidize it also but its activity is less than half that of the liver. Brain, muscle and blood are unable to oxidize choline under the conditions of our experiments.

Discussion. Page and Schmidt (5) showed some time ago that choline disappeared when incubated with liver. This disappearance has been shown to be due to the oxidation of choline by a specific enzyme in the liver of many animals. When choline is given in large quantities to animals part of it is eventually excreted in the urine as trimethylamine (6). The evidence presented in this paper shows that the liver enzyme in its attack upon choline is influenced by the hydrogen ion concentration and is able to oxidize choline further in alkaline than in acid solutions. The end product in an acid medium is a strong reducing agent and can form a compound with sodium bisulfite. In an alkaline medium more oxygen is taken up, no reducing substance can be found in the solution after oxidation and no bisulfite compound is formed. Further, synthetic betaine aldehyde is attacked by the enzyme in alkaline solution though in acid solution practically no extra oxygen is taken up. These facts indicate that betaine aldehyde is the end product in an acid solution and that in an alkaline solution the oxidation proceeds until betaine is formed. The measured oxygen consumption in all cases corresponds exactly to that necessary for the formation of these compounds. In the intact animal it is likely that this mechanism is one of many involved in the destruction of choline and that the betaine thus formed is then attacked further and possibly decarboxylated. No evidence has been obtained in isolated systems for the destruction of betaine. The function of the choline oxidase in liver may be one of protection against accumulation of choline and thus it may play a part in fat metabolism.

SUMMARY

1. The choline oxidase of rat liver has been purified.

2. The evidence indicates that at pH 6.7 choline is oxidized to betaine aldehyde with the uptake of one atom of oxygen and at pH 7.8 to betaine with the uptake of two atoms of oxygen. Synthetic betaine aldehyde is oxidized at pH 7.8 with the uptake of one atom of oxygen.

3. The enzyme can be separated from the alcohol oxidase of liver. It is also present in rat kidney but absent in blood, brain and muscle.

REFERENCES

(1) BERNHEIM, F. AND M. L. C. BERNHEIM. This Journal **104:** 438, 1933.
(2) TROWELL, O. A. J. Physiol. **85:** 356, 1935.
(3) CLIFT, F. P. AND R. P. COOK. Biochem. J. **26:** 1788, 1932.
(4) VOET, R. Bull. Soc. Chim. **45:** 1016, 1929.
(5) PAGE, I. H. AND E. SCHMIDT. Ztschr. Physiol. Chem. **191:** 262, 1930.
(6) GUGGENHEIM, M. AND W. LÖFFLER. Biochem Ztschr. **74:** 208, 1916.

A STUDY OF THE EFFECT OF VASCULAR CHANGES IN THE LIVER AND THE EXCITATION OF ITS NERVE SUPPLY ON THE FORMATION OF BILE

CARLOS A. TANTURI[1] AND A. C. IVY

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago

Received for publication July 12, 1937

It is generally believed that an increase in blood flow through the liver *per se* increases the formation of bile. This view is based chiefly on the observation of Heidenhain (1), who reported an increase in bile flow following splanchnic nerve section, and of Munk (2), who observed a decrease in bile flow on splanchnic stimulation. On the contrary, it has been found by using the thermostromuhr that an increase in flow through the liver occurs after the ingestion of a meal (3); and yet, several reports have appeared (4) in which it is noted that the ingestion of a meal failed to cause an increase in the formation of bile. Further, Lundberg (5) and Hillyard (6) claim that no significant change in bile formation occurs after sectioning the nerve supply of the liver, which is surprising in view of the vasoconstrictor action of the generous nerve supply of the organ. We have therefore repeated and extended the older experiments on the effect of section and stimulation of the nerve supply of the liver on the formation of bile. The results have caused us to investigate also the relative importance of the hepatic and portal blood supply, of increased portal pressure and blood flow, and of an increase in carbon dioxide and a lack of oxygen on the formation of bile.

METHODS. The experiments were performed on vigorous, fasting (24 hr.) dogs anesthetized with sodium pentobarbital, the anesthetic being replenished as required throughout the duration of the experiment. The carotid blood pressure was recorded; the cystic duct was obstructed and the common bile duct cannulated. To obtain a true control flow of bile it was found necessary to wait some ten or twenty minutes after the cessation of the operative manipulations in the abdomen. In all the dogs used in this study, except as otherwise stated, the control flow amounted to a rate of from 50 to 100 cc., or from 3 to 6 cc. per kilo body weight of bile per day, which is in the range of the low normal, fasting rates for a chronic biliary fistula dog. It should be added that as the experiment

[1] Becado, Comision Nacional de Cultura, Buenos Aires.

progressed over a period of several hours the rate of bile flow tended to decrease. When the nerves were stimulated a Harvard inductorium receiving current from two dry cells was used; a current of moderate strength denotes one when the secondary coil was set at about 6.5 cm.

RESULTS. *Stimulation of nerves. Section of the splanchnic nerves:* The splanchnics were sectioned as they emerged beneath the diaphragm in eleven animals. In nine of the eleven, the bile flow was definitely increased, confirming Heidenhain (fig. 1, 3 *B*). The degree of decrease in blood pressure was not correlated with the increase in bile flow. In one dog the fall in blood pressure (136 to 120 mm. Hg) amounted to only 16 mm. Hg, whereas a 40 per cent increase in bile flow occurred. The average increase in the rate of bile formation was 66 per cent, or an increase from 4.8 cc. per hour to 8.0 cc. The increase in rate of flow continued for at least 5 to 8 hours, although the flow tended to decrease as time elapsed.

In two experiments in which bile flow was stimulated with sodium dehydrocholate, given intravenously and continuously, section of the splanchnics resulted in a 33 and a 50 per cent increase in bile formation.

In five of seven animals from which the gastro-intestinal tract, spleen and pancreas had been removed, section of the splanchnics increased bile flow (fig. 1, 4 *C*).

It was observed that bile may be spontaneously formed when the systemic blood pressure is very low (20-40 mm. Hg). Bile flow ceased immediately after death; a postmortem flow of more than 6 drops (0.3 cc.) was never observed (fig. 1, 2 *A* and *B*).

Stimulation of the peripheral end of the right splanchnic: The peripheral end of the right splanchnic nerve was stimulated with currents of different strength for a period of five minutes or longer. We were more interested in obtaining a standard rise in blood pressure than in being particular concerning the strength of current used. On using a strong current and obtaining a 60 to 80 mm. Hg rise in pressure (i.e., above the control level), the bile flow was uniformly decreased (8 expts. in 6 animals) and almost ceased in some animals (fig. 1, 3 *E*). On using a weak current and obtaining a 10 to 20 mm. Hg rise in blood pressure, no change in bile flow resulted in three animals, an increase in three (5 tests) and a decrease in two (4 tests). These changes in rate of flow were directly correlated with changes in blood pressure. If the blood pressure was low (40 to 50 mm. Hg), a weak stimulation, resulting in a rise in blood pressure of from 10 to 15 mm. Hg, was associated with a slight increase in rate of flow of bile. If the blood pressure was higher, or if the pressure rose 15 to 25 mm. Hg, then a decrease in bile flow resulted, which was sometimes preceded by a temporary one to three drop increase in flow. On using a current of moderate strength, or one which caused a rise of 40 or 50 mm. Hg in

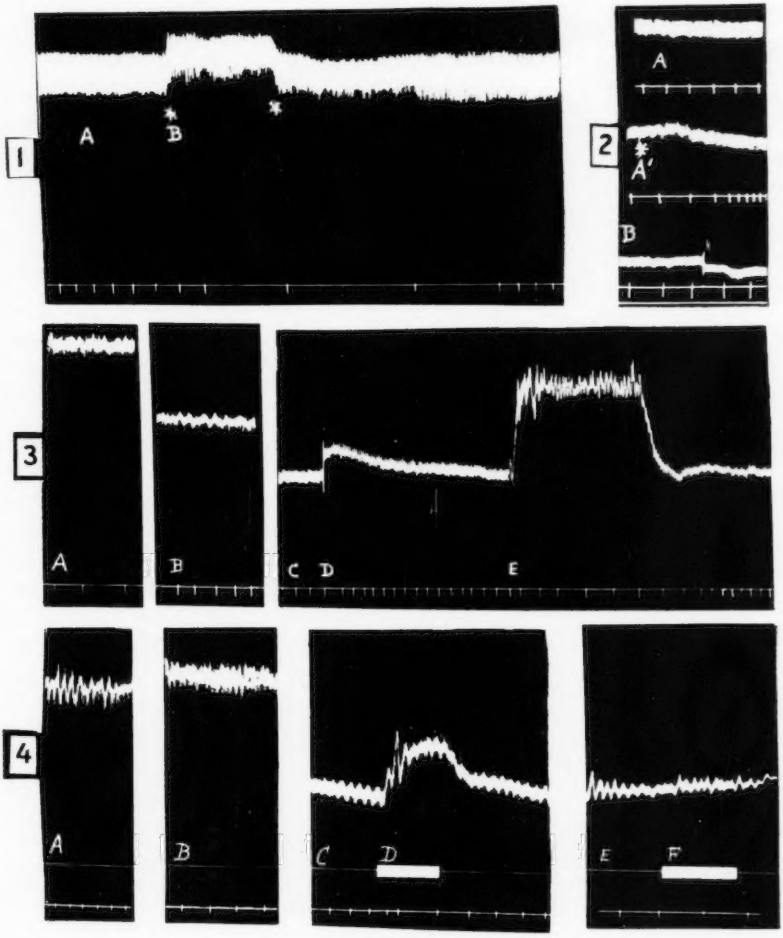


Fig. 1. The tracings show the blood pressure record and the drops of bile. Tracing 1: Shows the effect of stimulation of the hepatic nerves peripherally with the hepatic artery occluded. The nerves were stimulated at *B*; the artery was occluded at a point not shown on the tracing. Tracing 2: Showing the spontaneous secretion of bile when the blood pressure is very low in two different dogs A and B. At *A'* 5 cc. of 4 per cent dehydrocholate were injected intravenously. The rate of the spontaneous secretion in dog A is 3.0 cc. per hour. Tracing 3: *A*, control flow; *B*, flow after section of the splanchnic nerves; *C*, flow after dissecting but not cutting the hepatic nerves; *D*, occlusion of the hepatic artery; *E*, stimulation of the right splanchnic nerve peripherally with the hepatic artery still obstructed. Tracing 4: *A*, control flow; *B*, flow after evisceration, except liver; *C*, splanchnics sectioned; *D*, stimulation of right splanchnic peripherally; *E*, flow after section of hepatic nerves; *F*, stimulation of right splanchnic.

carotid blood pressure, the flow decreased promptly (4 animals), which in most instances (3 animals) was preceded by a temporary one to three drop increase in flow. When the flow of bile was stimulated with bile salt, stimulation of the splanchnic (3 animals) with any strength of current decreased bile flow, the one or two drop temporary increase being observed.

Exclusion of the adrenals did not modify the response. This procedure was introduced because epinephrine or ephedrine in any dose which caused a rise in blood pressure decreased the flow of bile. Histamine in doses which depress the blood pressure decreased bile flow during the period of the depression of the blood pressure; the same was true of amyl nitrite.

The increase in flow, which resulted occasionally when a slight rise in blood pressure was obtained in the presence of an initially low blood pressure, was due to a true increase in the formation of bile because the increase was not followed by a compensatory decrease in flow. The very temporary increase in flow that occurred frequently when the currents of moderate or stronger strength were used, was obviously due to a "squeezing out" effect. The subsequent decrease and almost cessation of flow was due to a true diminution of bile formation, because in no instance was the decrease in flow followed by a compensatory increase at the end of a five or seven minute period of stimulation of the nerve. After the end of such a period of stimulation the bile flow would return to normal within one to ten minutes.

Stimulation of the peripheral end of the hepatic nerves: The hepatic plexus about the hepatic artery near its origin was carefully dissected, sectioned and the peripheral end placed in a shielded electrode (7 animals).

Section of the hepatic nerves augmented bile flow from 25 to 100 per cent. If the splanchnics had been previously sectioned, the augmentation of bile flow did not occur uniformly (fig. 1, 4 *E*).

Stimulation of the hepatic nerves with any strength of current adequate to cause a rise in blood pressure resulted uniformly in a decrease in bile flow, the stronger the stimulus, the greater the rise in blood pressure and the decrease in bile flow. In these experiments the blood pressure was increased from 10 to 50 mm. Hg.

Stimulation of the splanchnic nerve (7 expts.) with the hepatic nerves sectioned resulted in no change in bile flow when the rise in systemic pressure (10-20 mm. Hg) was not marked (fig. 1, 4 *F*). In three experiments in which the rise in blood pressure was marked, the bile flow occasionally increased during the early stage of the rise in blood pressure and then decreased. The increase simulated a "squeezing out" effect.

The vascular changes occurring in the liver in this experiment are obviously complex. According to Burton-Opitz (8), stimulation of the splanchnic first increases and then decreases portal flow. With the hepatic nerves sectioned, vasoconstriction does not occur in the liver

and liver volume decreases but slightly (7) due to some decrease in portal flow; the arterial flow would passively increase with the rise in systemic blood pressure. Thus, the preliminary increase observed could be attributed to a "squeezing-out effect" due to a temporary increase in portal flow and to the rise in pressure in the hepatic artery, the secondary decrease being due to a rise in pressure in the arterioles which exerts pressure on the bile capillaries reducing bile formation. This interpretation explains why no change in bile flow resulted in four experiments in which the rise in blood pressure was not marked, under which conditions no significant change in hepatic volume occurs because the increase in flow through the hepatic artery compensates for the decrease in portal flow. As will be seen later, a decrease in portal flow alone cannot account for the foregoing observations.

Stimulation of the splanchnic and hepatic nerves with the hepatic artery occluded or the portal blood flow excluded: When the hepatic artery was occluded (4 expts.) and the hepatic nerves stimulated (fig. 1, 1 *B*) the bile flow was decreased. The decrease was preceded by a one or two drop increase in flow in two instances. Stimulation of the splanchnic nerves with hepatic artery occluded (5 expts.) and the hepatic nerves intact caused a decrease in bile flow also (fig. 1, 3 *E*).

With the hepatic artery occluded stimulation of the splanchnic or hepatic nerves is known to decrease liver volume (7). When the splanchnic is stimulated with the artery occluded, portal blood flow is decreased due to general splanchnic constriction and the blood supply of the liver is decreased. When the hepatic nerves are stimulated with the hepatic artery occluded the general splanchnic area escapes constriction, but portal pressure is increased slightly due to blood vessel contraction in the liver (7). Thus, blood flow to the liver is rather markedly decreased and in addition the arterioles and venules by contracting may exert pressure on the biliary capillaries decreasing bile formation, which occurs because a compensatory increase in flow does not occur after cessation of the stimulation of the nerves.

Stimulation of the splanchnic nerve with the gastro-intestinal tract, spleen and pancreas removed and the adrenals excluded, that is, with all the portal blood flow excluded, caused a decrease in bile flow (3 expts.) (fig. 1, 4 *D*). Under similar conditions, stimulation of the hepatic nerves caused a marked decrease in bile flow (3 expts.).

Under the conditions of these experiments stimulation of the sympathetic nerves supplying the liver obviously causes a marked reduction in blood flow to the liver and in addition a contraction of the venules and arterioles, both of which factors may reduce bile formation. This interpretation appears to be much more reasonable than to postulate the existence of specific inhibitory nerves in the splanchnic and hepatic nerves for the formation of bile.

Summarizing. From the preceding results it is evident that section of the splanchnic or section of the hepatic nerves, with or without the splanchnics being sectioned previously, results in an increase in bile flow which is appreciable and continues for several hours. The outstanding effect of stimulation of these nerves is a decrease in bile flow. The decrease in flow on stimulation occurs either when the hepatic arterial supply is excluded or when the portal venous supply is excluded by partial evisceration (removal of gastro-intestinal tract, spleen and pancreas).

Experiments were then undertaken to ascertain if the foregoing observations might be explained solely on the basis of changes in blood flow and intrahepatic vascular pressure.

RESULTS. *Direct alterations of blood flow through the liver. Obstruction of the hepatic outflow:* To constrict the hepatic outflow the usual technique of passing a small rubber tube beneath the hepatic veins was employed, the veins to the left and one to the right lobes being included.

It was found that an obstruction of the venous outflow of the liver sufficient to cause the liver border to extend slightly more than one millimeter during a period of five minutes was required to cause a measurable decrease in bile flow. An obstruction that caused the liver border to extend 5 mm. caused approximately a fifty per cent decrease in bile flow. Complete occlusion resulted in a prompt cessation of bile flow. After a complete occlusion lasting for one minute bile flow promptly returned to normal.

These results confirm those of Rohrig (9) who found that constriction of the cava, which has a similar effect on the liver as occlusion of the hepatic veins, decreased bile flow.

Portal occlusion and exclusion: The portal vein was partially occluded by a suitable clamp. An occlusion amounting to 25 per cent of the diameter of the portal vein caused no change in bile flow. A 50 per cent occlusion which perceptibly cyanosed the intestine and caused a gradual decrease in blood pressure decreased bile flow by about 50 per cent. Complete occlusion promptly decreased bile flow which gradually decreased in rate until death occurred.

To obtain a more reliable index of the importance of portal flow on bile formation, the entire gastro-intestinal tract, the spleen and the pancreas were removed, care being taken not to kink the hepatic artery or the cannula in the common duct. The operation was performed only after a control bile flow had been established. This experiment was performed in twelve animals.

The average control rate of flow in the twelve animals was 4.3 cc. per hour. After the partial evisceration, i.e., all the portal blood flow being excluded from the liver, the average rate of flow was 2.1 cc. per hour, a reduction of 50 per cent. The flow remained at this reduced rate for

from 3 to 7 hours, or during the period of observation; the flow tended to decrease as the experiment progressed. The minimum reduction in bile flow amounted to zero per cent (1 animal) the maximum 90 per cent (1 animal). The operation caused no change in blood pressure in some animals (fig. 1, 4 *B*), the greatest reduction amounting to 25 per cent. In the dog whose bile flow was reduced 90 per cent, the operation raised the blood pressure from 180 mm. Hg to 205 mm. Hg.

Thus the complete exclusion of portal blood flow, as well as any humoral influence from the gastro-intestinal tract and other abdominal viscera, in these acute experiments reduced bile formation to an average extent of only 50 per cent.

The only quantitative data supplied by the literature on the effect of an Eck fistula on bile volume output are those of Whipple and Hooper (10), who made only a six-hour collection of bile from their Eck-biliary fistula animals. When their data are expressed in cubic centimeters per kilo per six-hour period, their four Eck fistula dogs secreted 3.7, 4.1, 4.7 and 2.5 cc. of bile and their control animals 5.0, 4.3, 4.2, 4.4, 3.6 and 3.5 cc. Thus, only one of their Eck fistula animals secreted less bile than their control animals and that animal did not have a wide-open Eck fistula at autopsy. Ore (11) and Schiff (12) found that gradual obliteration of the portal vein does not prevent bile formation, and several authors (13) have observed that the liver will form bile as long as its arterial supply is intact. Hence, our observation that complete exclusion of the portal flow in acute experiments decreases the volume output of bile to an average extent of only 50 per cent is not surprising.

Occlusion of the hepatic artery: The hepatic artery was clamped near its origin both with and without section of the splanchnic and hepatic nerves and with and without the portal blood supply intact. It should be mentioned that occlusion of the hepatic artery near its origin does not completely deprive the liver of its arterial supply as long as the pancreatoduodenal vessels are intact.

When the liver is deprived of its portal supply by "evisceration," occlusion of the hepatic artery promptly diminishes bile flow, which ceases entirely after five minutes (4 expts.). On releasing the clamp on the artery after 30 minutes of occlusion, bile flow is resumed in about 20 minutes, and the rate of flow is approximately normal in 35 minutes (2 expts.). Thus, in so far as bile volume output is concerned the liver withstands "total" ischemia remarkably well.

With the portal supply intact, obstruction of the hepatic artery (7 animals) promptly and uniformly increased bile volume output. This increase persisted for from 2 to 5 hours, which is as long as we made observations. In two animals the flow began to decline after 2 hours. The increase in flow amounted to from 25 to 200 per cent. In three animals

the splanchnic nerves had been sectioned previously and in three they had not. After section of the splanchnics, the increase in flow on occlusion of the artery was not so marked (fig. 1, 3 *D*). Occlusion of the hepatic artery increased the blood pressure temporarily from 10 to 25 mm. Hg (fig. 1, 3 *D*).

After the hepatic nerves have been sectioned, which increases bile flow, occlusion of the hepatic artery further increased the rate of flow (4 animals). Section of the hepatic nerves with the artery clamped did not increase bile formation. The hepatic nerves, of course, could not be dissected from the hepatic artery without locally irritating the artery and causing it to constrict or without interfering with the nerves. These factors confused the interpretation of the results, which however show that obstruction of the artery increases bile flow to a greater extent than section of the nerves.

The literature on the effect of occlusion of the hepatic artery, which has been reviewed by Cameron and Mayes (14), agrees that the procedure does not prevent the formation of bile. But the experiments performed previously have not been done so as to reveal the augmentation in bile volume output noted above.

The effect of an increase in portal blood pressure and flow on bile formation: Two methods were employed to ascertain the effect of an increase in portal pressure and flow on bile formation. The *first method* consisted in connecting either the carotid or femoral artery by a gum rubber tube with the splenic branch of the portal vein, the animal having been given heparin which had no effect on bile flow. The *second method* consisted in irrigating the colon through the appendix with warm water (45°C.), which was done to augment the flow of blood through the colon and portal system.

On connecting the carotid or femoral artery (4 expts.) with the splenic vein, a decrease in bile flow occurred uniformly. The decrease in flow persists during a period of 15 or 20 minutes of arterio-portal anastomosis. This is also true when the liver is secreting copiously in response to bile salt (1 expt.). The flow of bile almost ceased entirely in one experiment in which the femoral pressure was 80 mm. Hg. A marked decrease occurred in two experiments in which the femoral pressure was 30 and 40 mm. Hg. When the hepatic artery is clamped, an arterio-portal anastomosis decreases bile flow (3 expts.). A compensatory increase in bile flow does not occur after removing the arterio-portal anastomosis. The results of this experiment show clearly that an increase in portal flow accompanied by a marked increase in portal pressure decreases bile formation.

With the idea of obtaining a more "normal" increase in portal blood flow as a result of splanchnic dilatation, the colon was irrigated continuously for about 20 minutes with tap water at 45°C. It has been shown

that application of heat to a loop of colon or intestine doubles or quadruples the blood flow through the loop (15). Undue distention of the colon was avoided by placing a large cannula in the rectum. While irrigating the colon care was exercised to avoid kinking the cannula in the bile duct. Four experiments were performed; and another in which the bile flow was augmented by the continuous intravenous administration of bile salt at a uniform rate. In all experiments the bile flow was decreased during an initial period of 5 or 10 minutes after the initiation of the irrigation. During this period the blood pressure was elevated (15–30 mm. Hg), indicating splanchnic stimulation. Then, the bile flow increased from 25 to 100 per cent above the control flow. After cessation of the irrigation bile flow returned to normal in from 5 to 15 minutes. This result shows that an increase in portal blood flow in the absence of vaso-constriction and of an increase in portal pressure augments the volume output of bile.

The effect of changes in the O_2 and CO_2 composition of the blood on bile formation: The following experiments were undertaken principally to ascertain whether the augmentation of bile flow that occurs following occlusion of the hepatic artery might be due to anoxemia. That such might be the case was not likely on the basis of the observation (vide supra) that bile may be formed when the blood pressure is very low and when the blood is definitely cyanotic.

The effect of changes in the O_2 and CO_2 composition of the blood was studied *a*, by having the animals rebreathe, and *b*, by having them breathe a mixture of 5 per cent CO_2 and 95 per cent O_2; *c*, of 10 per cent O_2 and 90 per cent N, and *d*, of 7.5 per cent O_2 and 92.5 per cent N.

The effect of asphyxia on liver volume has been studied by several investigators (7, 16, 17). In general, constriction followed by dilatation resulted. In the rebreathing experiments of Griffith and Emery (7) on anesthetized animals constriction of the liver was the usual result, while in those of Mattson (16) on unanesthetized animals dilatation was the only result.

Experiments were performed on five dogs subjected to rebreathing from a four liter and a sixteen liter tank. The extent of rebreathing was varied; the blood pressure and respiratory response was used as an index of the severity of the CO_2 increase and O_2 lack in the blood, sixteen different tests being performed. In no instance was the bile flow increased. The bile flow was generally decreased soon after the blood pressure started to rise, or when the liver first appeared to be congested (enlarged) by inspection (fig. 2). When the rebreathing was continued until a marked rise in pressure had been produced, and then terminated abruptly, as to obtain a marked fall in blood pressure with marked congestion of the liver, bile flow ceased for from three to fifteen minutes. In one instance an increase in bile flow resulted during the early period of marked congestion of the

liver associated with cardio-circulatory embarrassment. This was obviously a "squeezing-out" effect.

The 5 per cent CO_2 and 95 per cent O_2 and the other gas mixtures were administered by having the animal breathe for twenty minutes from a large tank filled with the mixture. Seven tests were performed on five animals. In one animal the bile flow was not influenced; in the others a 33 per cent (average) reduction in bile flow resulted. On two occasions when the bile flow was retarded, following the production of anoxemia (7.5 per cent O_2 + 92.5 per cent N), and when marked Traube-Herring waves were occurring in the presence of congestion of the liver, the administration of 5 per cent CO_2 and 95 per cent O_2 for five minutes corrected the circulatory state and resulted in the return of bile flow to normal.

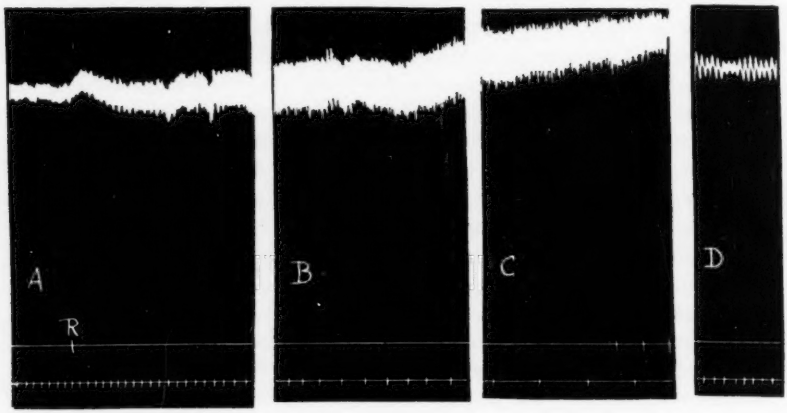


Fig. 2. Rebreathing; note the decrease in bile flow as the effects of rebreathing become more marked. *D*, the return to the control flow 5 minutes after cessation of rebreathing.

The mixtures of 10 and 7.5 per cent of O_2 with nitrogen were administered to four dogs seven times for twenty minutes. In three no effect on bile flow was observed. In one the bile flow was reduced by one-half after a period of about six minutes. The breathing of pure O_2 for a period of 5 or 10 minutes had no effect on bile formation.

The results show that the liver in so far as bile volume output is concerned is relatively resistant to anoxemia; at least anoxemia cannot account for the increase in bile flow that results from occlusion of the hepatic artery. These statements, of course, apply only to short periods. In regard to the effect of increased CO_2 content of the blood, it would appear that inhibition of bile flow is the result; but it is difficult to evaluate the results because of the sensitivity of bile formation and flow to vascular

changes in the liver. Actual determination of the CO_2 content of the blood flowing to and from the liver correlated with vascular changes in the organ would be required; the use of a combined London cannula and thermostromuhr technique would appear to be required to yield the most reliable data.

Discussion. It is clear from general observations made during these experiments that the liver will form bile in most instances as long as it receives blood, even when the systemic blood pressure is only 20 mm. Hg. In the presence of such a low blood pressure the intravenous injection of dehydrocholic acid will augment bile formation. This fact minimizes, if it does not annul, filtration pressure as a factor in the formation of bile.

Considerable stasis of blood in the liver (partial occlusion of the hepatic veins) is required to appreciably decrease bile volume output, although slight stasis will perceptibly influence it. Bile output returns to normal within forty minutes after a "total ischemia" of thirty minutes' duration. These observations and those made in the experiments dealing with anoxemia show that the liver, in so far as bile formation is concerned, is not very sensitive to anoxemia. Yet, bile flow ceases promptly on death, only from 0 to 6 drops being obtained after cessation of the heart beat when the bile was flowing briskly prior to death.

It is conceivable that vascular changes in the liver may modify the formation of bile *a*, by increasing or decreasing the minute volume flow of blood; *b*, by changes in filtration pressure in the capillaries and sinusoids; *c*, by changes in the CO_2 and O_2 content of the blood; *d*, by congestion or hyperemia of the liver with a resulting increase in intrahepatic pressure which overcomes secretory pressure of bile, or *e*, by an increase or a decrease in perilobular or lobular pressure which modifies the pressure in the bile capillaries and the branches of the interlobular ducts. Nerves may alter bile formation by causing vascular changes or by exerting a specific excitatory or inhibitory effect on the hepatic cells and the cells of the ducts, or by causing a contraction of the muscle fibers in Glisson's capsule. The outflow of formed bile may be influenced by changes in intrahepatic pressure exerted on the ducts or by the action of nerve impulses on the ducts, or on the muscle cells in Glisson's capsule.

The most important concept resulting from our observations is that the formation of bile is definitely influenced by the intrahepatic vascular pressure. An acute increase in portal and hepatic venous and arterial pressure decreases bile formation and, if of sufficient magnitude, will prevent bile from being formed. This is not surprising when one considers the fact that the secretory pressure of bile is relatively small (30 cm. H_2O) and the bile capillaries are surrounded by the cords of hepatic cells upon which the vascular pressure is exerted. In fact, bile formation is definitely decreased at an intra-common-ductal pressure of from 22 to 25 cm. of H_2O.

It should be pointed out that in our experiments a rise in portal and hepatic venous pressures was directly produced. This is not true for the hepatic arterial pressure. The statement that a rise in hepatic arterial pressure decreases bile flow is based on the observations that a sudden rise in systemic pressure with the hepatic nerves sectioned, and with the intestines out or in, decreases bile flow and that occlusion of the hepatic artery with or without the hepatic nerves sectioned increases bile flow.

The increase in bile formation following occlusion of the hepatic artery is best explained we believe, on a mechanical basis, as indicated in the preceding paragraph. The effect cannot be ascribed to anoxemia because anoxemia, asphyxia, or an increase in the CO_2 content of the blood, when effective, decrease bile formation. A rather remote possibility is based on the idea that the capillaries from the hepatic artery, which form a plexus (18, 19, 20) about the bile ducts, normally absorb water from the bile (18); hence occlusion of the artery would stop this process and result in a more copious flow of thin bile.

On a mechanical basis alone it is not clear why section of the hepatic nerves, which should result in dilatation of the hepatic arterioles and also of the portal venules (7, 8, 20), and occlusion of the hepatic artery, which should collapse the arterioles and decrease portal pressure (20), should both result in an increase in bile flow. If section of the hepatic nerves should decrease portal pressure, then the mechanical explanation would hold; but the literature provides no good evidence regarding this point. Any explanation has to consider the observation that occlusion of the artery increases bile formation more than section of the hepatic nerves, and that after section of the nerves occlusion of the artery further augments bile formation. These observations support a mechanical explanation; but section of the nerves may also increase blood flow through the liver to a significant extent, which may account for the augmentation of bile formation.

The fact that bile formation increases after section of the hepatic nerves and decreases when the nerves are stimulated may indicate the existence of true inhibitory secretory nerves. This is uncertain for the following reasons. Occlusion of the artery increases bile formation to a greater extent than section of the hepatic nerves, and both prodecures obviously produce vascular changes in the liver. Section of the hepatic nerves after occlusion of the artery does not significantly alter bile flow. Stimulation of the hepatic nerves distally definitely decreases bile formation, but also reduces both the portal and arterial supply of the liver and liver volume (7, 8, 20). The occlusion of the artery not only decreases the inflow of blood (average 30 per cent), but also decreases portal pressure (20) and the pressure in the sinusoids.

An increase in blood flow through the liver augments bile output in

acute experiments, except when the increase in blood flow is associated with an increase in intrahepatic vascular pressure. The only direct evidence upon which this tentative conclusion rests is that obtained from the arterio-portal anastomosis. The conclusion is supported by considerable indirect evidence, such as the results of the colon irrigation experiments, of splanchnic and hepatic nerve section, etc. We attempted to obtain direct evidence by introducing heparinized blood into the splenic vein at a constant rate and at a pressure only slightly above that of the portal vein (10 cm. of blood). But, any pressure (e.g., 15 cm.) adequate to give an appreciable inflow of blood (30 cc. per minute), decreased bile formation. It would appear that satisfactory direct evidence may only be obtained through the direct measurement of pressures by a needle technique and of blood flow with a thermostromuhr. Such a study would also control the unknown factor of the "sluice mechanism" located in the hepatic veins near the caval orifices in the dog (21).

GENERAL CONCLUSIONS

1. The formation of bile is definitely influenced by the intrahepatic vascular pressure. An acute increase in portal and hepatic venous or hepatic arterial pressure decreases bile formation. Occlusion of the hepatic artery augments bile volume output at least for a few hours. This might be expected on the basis of the relatively low secretory pressure of bile, and the exposure of the bile capillaries to the effects of changes in vascular pressure.

2. Stimulation of the sympathetic nerve supply of the liver decreases bile formation; the evidence indicates that the decrease is due to vascular or mechanical changes in the liver rather than to true inhibitory secretory nerves.

3. It is tentatively concluded that an increase in blood flow through the liver augments bile output in acute experiments, except when the increase in blood flow is associated with an increase in intrahepatic vascular pressure.

REFERENCES

(1) Heidenhain. "Herman's Handbuch," vol. 5, p. 266; also Studien physiol. Inst. Breslau **4**: 226, 1868. Ananassiew. Pflüger's Arch. **30**: 385, 1883.
(2) Munk. Pflüger's Arch. **8**: 151, 1874.
(3) Herrick, Essex, Mann and Baldes. This Journal **109**: 52; ibid. **108**: 621, 1934.
(4) Babkin. Die Aussere Sekretion der Verdannugsdrusen, 1928.
Koster, Shapiro and Lerner. This Journal **115**: 23, 1936.
(5) Lundberg. This Journal **98**: 602, 1931.
(6) Hillyard. This Journal **98**: 612, 1931.
(7) Griffith and Emery. This Journal **95**: 20, 1930.
Macleod and Pearce. This Journal **35**: 87, 1914.
(8) Burton-Opitz. J. Physiol. **4**: 103, 115, 1911; **5**: 83, 189, 309, 329, 1912; **7**: 57, 1914.

(9) ROHRIG. Loc. cit. SCHAFFER. Textbook of physiology, London, 1900.
(10) WHIPPLE AND HOOPER. This Journal **42:** 545, 1917; ibid. **43:** 290, 1917.
FOSTER, HOOPER AND WHIPPLE. J. Biol. Chem. **38:** 393, 1919.
VOEGTLIN AND BERNHEIM. J. Pharmacol. Exper. Therap. **2:** 455, 1910–11.
(11) ORE. J. d. Anat. et d. l. Physiol. **1:** 565, 1864.
(12) SCHIFF. Pflüger's Arch. **3:** 598, 1870.
(13) BETZ. Sitz. d. math. Klasse d. Kais. Akad. d. Wissensch., Wien. **46:** 238, 1863.
COLOSANTI. Bull. Accad. med. di Roma **27:** 487, 1896.
ASPE. Loc. cit. SCHAFFER. Textbook of physiology, London, 1900.
(14) CAMERON AND MAYER. J. Path. Bact. **33:** 799, 1930.
(15) BEAZELL, SCHMIDT AND IVY. This Journal **119:** 197, 1937.
(16) MATTSON. This Journal **90:** 146, 1929.
(17) NEUBAUER. Biochem. Ztschr. **43:** 335, 1912; ibid. **52:** 118, 1914.
FRANCOIS-FRANK AND HALLION. Arch. Physiol. Norm. et Path. **9:** 434, 1897.
(18) HIGGINS. Anat. Rec. **32:** 89, 1926.
(19) OLDS AND STAFFORD. Johns Hopkins Hosp. Bull. **47:** 176, 1930.
(20) MCMICHAEL. J. Physiol. **75:** 241, 1932.
(21) BAUER, DALE, POULSSON AND RICHARDS. J. Physiol. **74:** 343, 1932.

THE PART PLAYED BY REFLEXES FROM THE CAROTID BODY IN THE CHEMICAL REGULATION OF RESPIRATION IN THE DOG[1]

J. H. COMROE, JR. AND CARL F. SCHMIDT

From the Laboratory of Pharmacology, University of Pennsylvania

Received for publication July 12, 1937

That respiration can be profoundly influenced by reflexes set up in the region of the carotid sinus by chemical agents may now be regarded as an established fact. The original observations of Heymans and his collaborators (Bouckaert, Dautrebande, Regniers, 1930, 1931, 1933) have been repeated and confirmed by a number of workers (Owen and Gesell, 1931; Selladurai and Wright, 1932; Schmidt, 1932; Gemmill and Reeves, 1933; Gayet, Bennati, and Quivy, 1935; Green and de Groat, 1935; von Euler and Liljestrand, 1936; Winder, 1937; Smyth, 1937). Further confirmation of a new sort has been furnished by studies of action potentials in the sinus nerve elicited by changes in the chemical composition of the blood in the carotids (Heymans and Rijlant, 1933; Bogue and Stella, 1935; Samaan and Stella, 1935; Zotterman, 1935). Although Gollwitzer-Meier and Schulte (1931) were unable to confirm any of Heymans' results, in subsequent experiments (Gollwitzer-Meier, 1934) evidence was obtained of the presence in the carotids of a reflex mechanism reacting to at least one chemical agent—lobeline.

The information now available on this subject is however incomplete or conflicting in two important respects. One is the location of the chemically sensitive receptors, the other the physiological importance of the reflexes arising from them. With regard to localization, Heymans and his co-workers have believed from the start that the carotid body (glomus caroticum, ganglion inter-caroticum, paraganglion caroticum, glomerulus intercaroticus, glandula carotica) contains all of the chemically sensitive receptors of the carotid system, as de Castro (1928) had suggested on morphological grounds. They (Bouckaert, Regniers, and Heymans, 1931) were able to abolish the sensitivity of the carotids of dogs to pressure without removing the response to chemical agents and (Heymans and Bouckaert, 1933) to inactivate the chemically sensitive receptors without

[1] A preliminary report of these experiments was made at the meeting of the American Physiological Society at Memphis, Tenn., on April 24, 1937 (This Journal **119**: 290, 1937).

abolishing pressure reflexes. They concluded that the chemical receptors are located cephalad to the carotid sinus in the dog, probably in the carotid body. A similar conclusion was reached by Schmidt (1932), but whether the chemical receptors lie in the occipital artery or in the carotid body, which gets its blood supply from that artery in the dog, remained uncertain. Gollwitzer-Meier (1934) believed that the receptors reacting to lobeline lie in the occipital artery and not in the carotid body. The point is important because the carotid body is the first of the small aggregations of so-called "chromaffine tissue"[2] to which a definite function has been ascribed, and it turns out to be a very unusual and hitherto unsuspected one.

As for physiological importance, there is general agreement that the hyperpneas produced by anoxemia and by certain chemical agents (cyanide, sulphide, nicotine, lobeline) are largely if not entirely reflex in origin, but concerning the effects of CO_2 there is considerable difference of opinion. Heymans et al. believed that although the chemical receptors can respond to changes in CO_2 tension the response of the whole organism is due much more to direct effects on the center than to reflexes. This view is shared by Schmidt (1932), by Gemmill and Reeves (1934), and by Smyth (1937); these workers concluded that carotid reflexes play no essential part in the response to hypercapnia although they are the major factor in the hyperpnea of anoxemia. On the other hand, Selladurai and Wright (1932), Gayet, Bennati, and Quivy (1935), Samaan and Stella (1935), Green and de Groat (1935), and von Euler and Liljestrand (1936) believed that all of the physiological responses to CO_2 involve carotid reflexes to a considerable degree. There is little conclusive evidence concerning the relative sensitivity of the cells of the center and of the reflex receptors to changes in the CO_2 tension of the blood as well as concerning the parts played by the two structures in the total response of the animal to CO_2.

The purpose of our experiments was first to localize the chemically sensitive receptors in the carotids as accurately as possible and then to utilize the information so obtained in a further study of the relative importance of carotid reflexes and of direct central effects in the respiratory responses to CO_2, which means in the normal regulation of breathing.

GENERAL METHODS. Dogs were used exclusively, largely because of the readiness with which an anatomical and functional separation of the pressure and chemical receptors of the carotids can be accomplished in this animal. Routine procedures in all experiments were anesthesia by morphine (2

[2] We realize that the carotid body of man and other mammals is now known not to show typical chromaffine reactions but the term chromaffine has been extensively used to designate these structures and none of the alternatives (paraganglion, glomus, body, gland) is free from objections.

mgm. per kilo subcutaneously) and chloralose (70–100 mgm. per kilo intravenously), recording of respiratory movements by a pneumograph, measurement of respiratory minute volume by a gas meter attached to the expiratory side of a valved tracheal cannula, and recording of femoral arterial blood pressure by a mercury manometer using 25 per cent sodium thiosulfate as the anticoagulant. In all experiments both depressor nerves were cut, either as separate structures when both could be isolated and identified, otherwise by section of one or both vago-depressor trunks. Division of the depressor nerves was regarded as complete when blood pressure remained at a level above 200 mm. as long as both carotids were clamped.

1. *Localization of the chemically sensitive receptors. A. Anatomical features of the carotid body in the dog.* The structure is grossly visible as a reddish nodule firmly attached to the mesial surface of a very small branch which arises either from the occipital artery near its origin from the external carotid, or from the latter vessel itself (fig. 1). The carotid body is directly in the pathway of the nerve fibers from the carotid sinus as these pass over the occipital artery on their way to join the glossopharyngeal nerve near the jugular foramen. Being thus completely surrounded by fibers of the sinus nerve it appears to be several times larger than its actual size (1–1.5 mm. in diameter). The artery to which it is attached supplies it with blood through several channels which appear as tiny openings in the arterial wall at the level of the organ[3]; the artery then runs onward to break up in the tissues of the neck, where its branches anastomose freely with those of other arteries. These arterial relations are important from the standpoint of physiological experiments because they make it impossible to abolish the circulation through the carotid body simply by clamping its afferent artery; blood can still reach it by way of anastomoses with the distribution of other occipital branches, including a small branch which runs with the sinus nerve, and the latter branch cannot be occluded without damaging the nerve. In the perfusion experiments which will be described later we found that the pressure existing in these anastomotic pathways was practically equal to systemic arterial pressure, and were able to secure consistent results only when the perfusion pressure was higher than systemic; if the systemic pressure was the higher, changes in chemical composition of the perfusing blood were without effect because

[3] The small artery supplying the carotid body loses most of its muscular coat and assumes a vein-like appearance on the surface to which the body is attached. On this surface the vessel communicates with the body by several small channels. Beyond the body the vessel regains its arterial character (Addison and Comroe, 1937). As far as we know at present, this small artery serves as both afferent and efferent vessel for the carotid body; no venous channels leading from the latter have as yet been found. Further studies are being made.

the carotid body then received unaltered blood from the systemic circulation. This anatomical peculiarity may explain some of the negative results obtained in perfusing the carotids with anoxic blood. Furthermore, the hyperpnea of anoxemia cannot be prevented by occluding both occipital arteries proximally to the carotid bodies, and even when the arteries are also occluded distally blood from the systemic circulation can still reach the bodies through the tiny arteries which run with the sinus nerves.

The above-mentioned anatomical relationships are illustrated in figure 1.

B. *Exclusion of the chemically sensitive receptors by clamping the arterial supply of the carotid body.* For this purpose we made use of a procedure employed by Heymans et al. (1930), namely, injection of a minute dose of lobeline or cyanide into the common carotid artery. The results were clean-cut and decisive (fig. 5): the brief but intense hyperpnea which normally results, was entirely absent when the injection was repeated while the small artery to the carotid body was clamped, but when the artery was reopened the injection was again effective. When the clamp was placed upon the occipital artery so that it occluded the latter but not the branch to the carotid body, the hyperpnea produced by the injection was not diminished; it may even have been intensified. Similar results were obtained with cyanide. Section of the sinus nerve entirely abolished the effects of the injections. We conclude that the receptors responsible for the hyperpnea produced by intracarotid injections of lobeline and cyanide are located in the carotid body and not in the first portion of the occipital artery; the effect of denervation shows that an action upon the centers played no part in these results.

Attempts to duplicate these observations using systemic anoxemia as the stimulus failed for reasons that have been explained in the section on the anatomical relations of the carotid body.

C. *Absence of chemically sensitive receptors in the carotid sinus.* Additional evidence that the chemical sensitivity is limited to the carotid body was obtained by the technic illustrated in figure 2. The reactivity of the pressure receptors of the innervated carotid was demonstrated by the occurrence of hypertension when that carotid was clamped.

With this preparation a chemical excitant injected into the denervated carotid, *A*, reached the innervated carotid body but not the carotid sinus; injected into the innervated carotid, *B*, it reached the carotid sinus but not the carotid body. A typical result is illustrated in figure 6. Injection of lobeline at *B* failed entirely to stimulate breathing, but the same injection at *A* caused characteristic hyperpnea. Section of the sinus nerve entirely abolished the latter effect. These results show that the receptors of the carotid sinus are insensitive at least to one characteristic chemical excitant and justify the belief that they are pressure receptors only. As

will be seen later, the receptors of the carotid body are insensitive to pressure. There is therefore a complete anatomical separation of the two sets of receptors in the carotid of the dog.

2. *Reactivity of the carotid body to changes in O_2 and CO_2 content of the blood.* The procedure just described was modified so as to permit perfusion of the innervated carotid body independently of the carotid sinus and testing of the reactivity of the carotid body to the physiological excitants

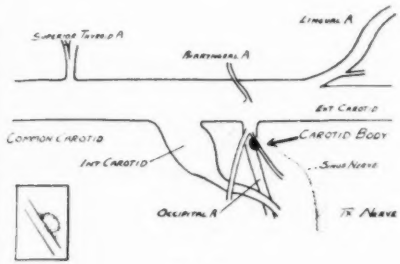


Fig. 1

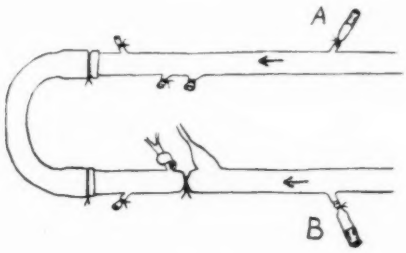


Fig. 2

Fig. 1. Anatomical relations of right carotid sinus and carotid body of the dog (viewed from mesial surface).

Dots indicate course of sinus nerve from its origin on the carotid sinus walls until its entrance into skull. The small artery to which the carotid body is closely applied can be occluded without damage to the body or nerve.

Inset shows relation of carotid body to its artery. Note the tiny channels in the arterial wall leading to the carotid body.

The superior thyroid artery was utilized for intracarotid injections.

Fig. 2. Method used in separating chemical and pressure receptors in the carotid of the dog.

Carotid *A* was denervated by completely freeing it from all surrounding tissues between the origins of the superior thyroid and lingual arteries. External carotid *A* was anastomosed to external carotid *B* with the aid of Payr cannulae and an artery from another dog. Innervation of carotid *B* remained intact but all branches of the external carotid were tied except the occipital. A ligature was tied firmly about the external carotid between the occipital and internal carotid arteries, avoiding damage to the sinus nerve. A cannula was placed in each superior thyroid artery for intracarotid injections of lobeline or cyanide.

provided by changes in the gaseous composition and pH of the blood. A diagram of the technic employed is shown in figure 3.

As perfusion fluids we employed Locke's solution or fresh dog's blood, defibrinated or heparinized, maintained at a temperature of 38 to 40°C. By means of the apparatus pictured (fig. 3), the O_2 content of blood could be altered as much as 14 vols. per cent and CO_2 content changed by 31 vols. per cent within a minute or two. Gas analyses were made by the manometric method (Van Slyke and Neill) on blood samples collected

from a side arm of the inflow cannula or from the outflow. Reactivity of the carotid body was tested with α lobeline immediately before and again after perfusion was begun. Those experiments were discontinued

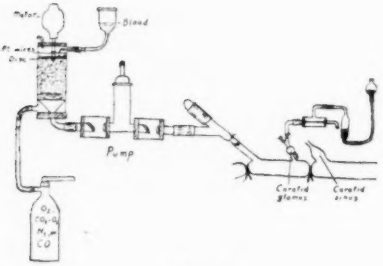


Fig. 3

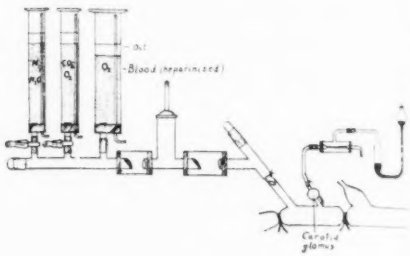


Fig. 4

Fig. 3. Method used to perfuse a carotid body with fluids of varying gas content.

As blood or Locke's solution enters the vertical cylinder from reservoir above, it is spread as a thin film along the sides of the cylinder by a motor-rotated celluloid disc. Its gas content can be rapidly changed by bubbling through the cylinder O_2, CO_2-O_2, N_2, or CO from the tank below. Excessive frothing is controlled by platinum wires attached to the rotating disc.

Using the Drinker-Richards pump, blood with its gas content thus altered is now perfused through the carotid body via a cannula in the lingual artery. The external carotid is ligated beyond the lingual artery and again between the occipital and internal carotid arteries. All other carotid branches are ligated except the occipital. Outflow is collected from a branch of the occipital beyond the carotid body and passed through a "peripheral resistance" attachment to maintain perfusion pressure above systemic blood pressure.

The carotid sinus, though still functioning normally, is excluded from the perfusion system.

Fig. 4. Method for perfusion of carotid body with animal's own blood (animal used as tonometer).

Blood samples were collected under oil in graduated cylinders from the dog's femoral artery, and heparinized. The first was collected during O_2 inhalation, the second at the height of hyperpnea produced by inhalation of 10 per cent CO_2 in O_2, and the third during N_2O inhalation. These three cylinders were then inverted and attached in succession to the pump intake. Control (O_2), hypercapnic, or anoxic blood could then be alternately perfused through the carotid body of the same dog, without interruption of the flow or change in pressure. The preparation of the artery is the same as in figure 3. Changes in rate and minute volume of breathing were recorded both during the inhalation and during the subsequent perfusion of the carotid body with the blood collected during the inhalation.

in which little or no response to lobeline indicated damage to the nerve or blood supply of the carotid body.

In 32 technically successful experiments, reduction in the O_2 content of the blood or Locke's solution used to perfuse the carotid body caused some degree of hyperpnea; in 17 of these, similar results were also obtained

by increasing the CO_2 content. Denervation of the carotid body, by actual section of the sinus nerve or by injection of 1 per cent procaine into it, uniformly abolished such hyperpneas, showing that they were not due to access of altered blood to the respiratory center. This control procedure was necessitated by our inability to secure an isolated perfusion system without damaging the nerve and blood supply of the carotid body.

Examples of the results obtained in this series of experiments are shown in figure 7. It is evident that a reflex hyperpnea can be elicited by perfusing a carotid body with blood which is either rich in CO_2 or poor in O_2. Similar results were obtained with Locke's solution. The question of major interest—the part played by such reflexes in the response of the whole animal to CO_2—remains unanswered, however, for the changes in CO_2 content of the blood required to elicit reflex hyperpnea in these experiments were beyond physiological limits. In the example shown the CO_2 content of the perfusing blood was increased from 3.2 to 34.5 vols. per cent and respiratory minute volume increased 93 per cent, which was an unusually marked effect. Reduction in O_2 content from 21.1 to 14.4 vols. per cent increased respiration 150 per cent in the same animal, and this in the face of a marked reduction (from 18.6 to 3.1 vols. per cent) in the CO_2 content of the perfusing blood. These experiments therefore simply confirmed the conclusions drawn from an earlier study (1932) but did not enable us to decide the part played by reflexes in the total respiratory effects of either CO_2 or anoxemia. For that purpose it was necessary to compare the reflex (carotid body) response with that elicited by passage of the same blood through the respiratory center, and to keep the changes within limits known to be physiological. Experiments of this nature are described in the next section of this paper. First it may be of some interest to mention a few observations of the effects of using carbon monoxide as the gas to which the blood was exposed in the equilibrating cylinder in the experiments now under consideration.

This was done in three experiments, the object being to determine whether CO has a direct action of its own upon the carotid body receptors or whether it acts merely by reducing the oxygen tension of the blood. In each case hyperpnea appeared when the monoxide-treated blood was pumped through the carotid body, the response differing in no way from that elicited by perfusion with fluids made anoxic by exposure to nitrogen. The interesting feature was that the hyperpnea ceased as soon as the blood was exposed to oxygen although the amount of oxygen taken up was negligible and the CO content of the blood was not measurably reduced.

The results of one typical experiment illustrate these points: When CO was bubbled vigorously through the blood in the equilibrating cylinder an intense hyperpnea occurred (rate from 9 to 15 per minute, minute volume from 3000 cc. to 9250 cc.). The oxygen content of the blood at this

point was found to have been reduced by 4.67 vols. per cent (from 11.67 to 7.00). While the hyperpnea was at its peak oxygen was bubbled vigorously through the blood in the cylinder. The hyperpnea ceased abruptly

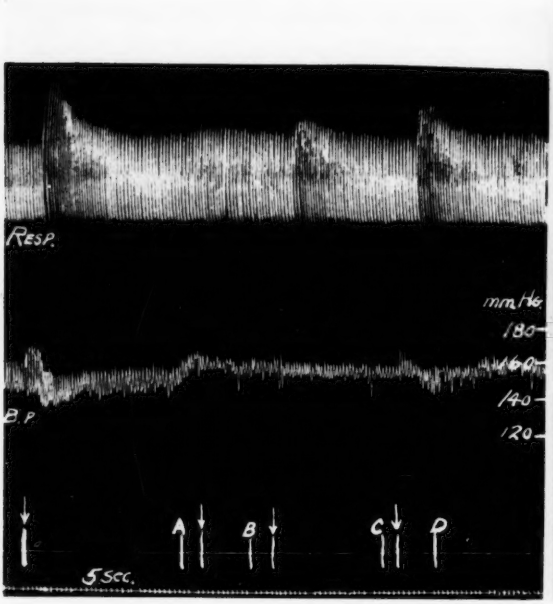


Fig. 5

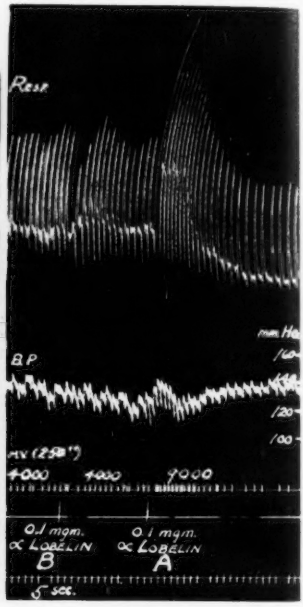


Fig. 6

Fig. 5. Localization of the chemically sensitive receptors in the carotid body. Dog. Chloralose. Each arrow indicates an injection of 0.1 mgm. α lobeline into the common carotid through a cannula in the superior thyroid artery. At *A*, the small artery leading to the carotid body was clamped near its origin from the occipital artery (see fig. 1). It remained clamped until released at *B*. From *C* until *D* the occipital artery was occluded just beyond the origin of the branch to the carotid body.

Fig. 6. Localization of chemically sensitive receptors in the carotid.

Dog. Chloralose. Prepared as in figure 2. From above downward: Respiration (pneumograph), femoral B. P., minute volume of respiration (each mark representing 250 cc. expired air), signal, time in 5 sec. intervals. Injection of 0.1 mgm. α lobeline in right carotid, *B*, no hyperpnea. Injection of 0.1 mgm. α lobeline in left carotid, *A*, intense hyperpnea.

though the oxygen content of the blood had increased only 0.42 vol. per cent and its monoxide content remained unchanged at approximately 8 vols. per cent. Since the hyperpnea was abolished by only a trifling increase in the oxygen content of the blood and before the CO had been

displaced from its combination with hemoglobin, it is evident that CO stimulates the carotid body only indirectly by reducing the oxygen tension of the blood. After perfusion with monoxide-containing blood the responses to lobeline and to blood rendered anoxic by nitrogen were as vigorous as before.

3. *Evaluation of the reflex and central factors concerned in the respiratory responses to physiological changes in the CO_2 and O_2 content of the blood.* The technic used in these experiments is illustrated in figure 4. The preparation was exactly the same as that described above for perfusion of one carotid body except for omission of the equilibrating cylinder. In

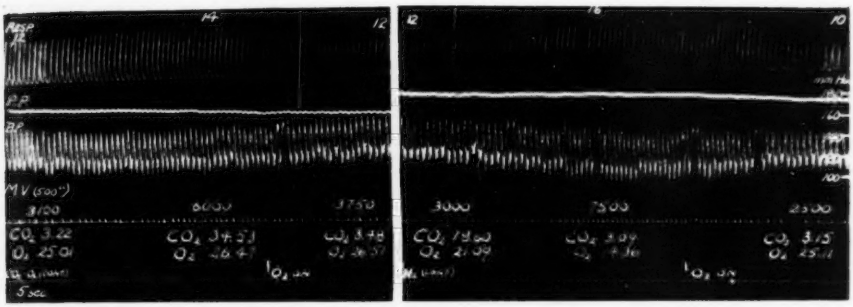


Fig. 7. Comparison of carotid body hyperpneas produced by hypercapnic and anoxemic bloods.

Dog. Chloralose. Right carotid perfused as shown in figure 3. From above downward: Respiration (pneumograph); perfusion pressure, *P.P.*; femoral blood pressure, *B-P*; respiratory minute volume, each mark indicating 500 cc. of expired air; CO_2 and O_2 content (vols. per cent) of perfusing blood; signal; time in 5 sec. intervals. Record on left shows hyperpnea produced by increase of 31.3 vols. per cent CO_2 (rate increased from 12 to 14; minute volume increased by 93 per cent); on the right is shown the hyperpnea induced by reduction of 6.7 vols. per cent O_2 (rate increased from 12 to 16; minute volume increased by 150 per cent despite extreme reduction in CO_2 content at the same time).

its place we used graduated cylinders of 100 to 250 cc. capacity which were fitted with two-holed rubber stoppers containing a short glass tube (for attachment to the pump intake) and a long tube (to admit air when the cylinder was inverted). At the start of the experiment the carotid body was usually perfused with oxygenated Locke's solution, and this could be resumed whenever necessary. While the animal was breathing oxygen about 200 cc. of blood were drawn from a femoral artery into one of the cylinders and mixed with heparin solution under oil. Then an inhalation of 10 per cent CO_2 in oxygen was given and at the height of the hyperpnea a second sample of 80 to 100 cc. of blood was collected in another cylinder. A third sample of similar size was collected in the third cylinder while

the animal was breathing nitrogen or nitrous oxide. Fresh heparinized blood from another dog was injected intravenously if necessary, but this was not usually the case since animals weighing more than 10 kilos were used in most of the experiments and the shed blood was eventually returned, either through unclosed arteries in the perfusion system or by injection into a femoral vein of the blood collected from the outflow cannula.

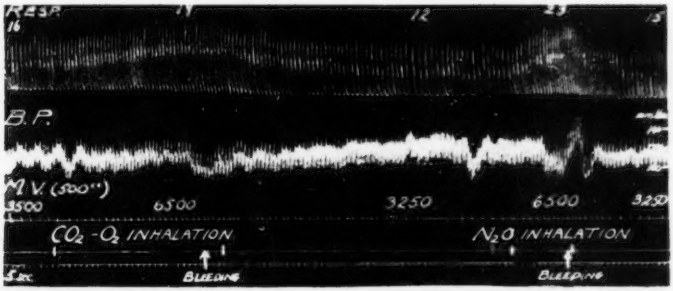
The blood samples collected in this way were then allowed to flow in succession through the pump intake, the larger (oxygen inhalation) one being used for perfusion before and after each of the others; the perfusion usually took place within 15 minutes after the collection. In this way we were able both to keep the changes in the perfusing blood within limits known to be physiological and to compare the animal's systemic response with the reflex effects of the same blood. In nine experiments in which a strong reflex hyperpnea ensued when hypercapnic or anoxic blood was perfused through the carotid body we reserved enough of each sample to enable us to repeat the perfusion after section of the sinus nerve; the hyperpnea was then entirely absent in each case.

An example of the results obtained is shown in figure 8. This animal showed an unusually marked reflex response to hypercapnic blood, although the reflex effect was considerably less than the systemic. The hyperpnea elicited by perfusion of anoxic blood through the carotid body was stronger than that from hypercapnic blood in this animal, and this was true practically without exception in all our experiments. In fact blood sufficiently hypercapnic to cause a definite hyperpnea at the time of the inhalation of CO_2 frequently failed to cause any reflex hyperpnea whatever, while blood anoxic enough to cause hyperpnea at the time of the inhalation (reduction in blood O_2 amounting to 4 vols. per cent or more) invariably caused a reflex hyperpnea when perfused through the carotid body. It was of course possible to control the degree of the hypercapnia or anoxemia by varying the length of the inhalation or by diluting the inhaled gas mixture, and it was soon evident that the reflex response to hypercapnia became more consistent and more marked as the degree of hypercapnia increased while that to anoxemia showed much less of the same tendency.

Technically satisfactory experiments of this sort were made on 14 animals. The criteria used for judging them satisfactory were definite hyperpnea from inhalation of CO_2; active respiratory response to injection of lobeline into the perfusion stream at the beginning and end of the observation period; at least one test of hypercapnia and of anoxemia; perfusion pressure well above systemic; patency of vessels in the perfused area, as evidenced by maintenance of a reasonably rapid perfusion flow without excessively high perfusion pressure; satisfactory analyses of the blood samples. A total of 41 observations was made of the systemic and

reflex effects of hypercapnia, 44 similar observations dealing with anoxemia. The results follow.

A. *Relation of the constancy of the reflex hyperpnea to the degree of hypercapnia or anoxemia.* Mention has already been made of the greater



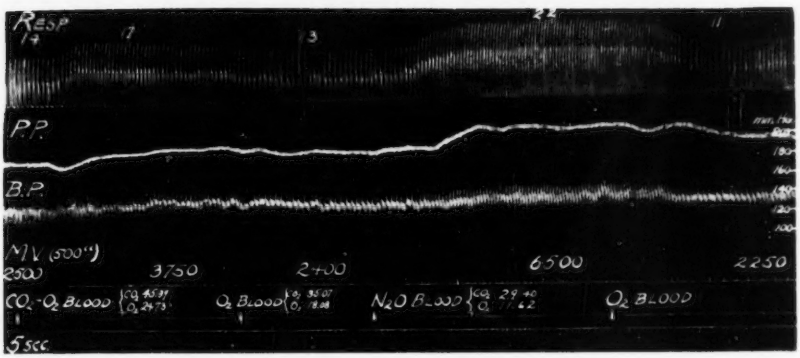


Fig. 8. Comparison of systemic and reflex (carotid body) hyperpneas produced by same blood in same animal.

At the height of hyperpneas produced by inhalation of CO_2 (10 per cent in O_2) or N_2O (upper record) blood samples were collected from the femoral artery under oil and heparinized. A few moments later, these two blood samples were perfused in succession through a carotid body of the same animal (lower record) after control perfusions with blood previously collected during inhalation of O_2. Note that the systemic response to CO_2 inhalation (upper left) is much greater than the reflex response to the same blood (lower left) whereas the reflex response to anoxia (lower right) is equal to the systemic response to anoxia (upper right). (See fig. 4 for method used.) In this experiment one carotid body was intact, the other perfused with oxygenated Locke's solution, during the inhalations.

frequency with which hypercapnic blood caused a reflex hyperpnea as the degree of hypercapnia became greater, and the absence of a similar tendency with anoxemia. This is illustrated in figure 9, in which the proportion of positive responses (hyperpneas) to the total number of observations

is shown; the results are grouped according to the extent of the change in gas content of the blood and the actual number of observations in each group is indicated at the top of the chart. No observations with anoxia amounting to less than 4 vols. per cent are included because changes of that order did not elicit any response, systemic or reflex, in our experiments and would not be expected to do so in the intact animal; observations made with increased CO_2 over the same range are included because there was definite hyperpnea during the inhalation in all cases. The intensity of the various hyperpneas is not considered here but will be in the following sections.

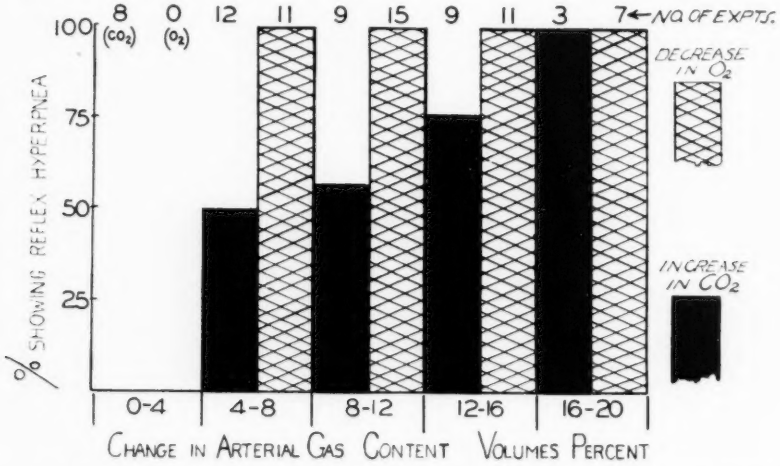


Fig. 9. Relative frequency of reflex hyperpneas produced by anoxic and hypercapnic bloods.

Frequency of reflex hyperpnea due to CO_2-excess increases as blood CO_2 content is increased, but only when CO_2 increases 16 to 20 vols. per cent is there consistent reflex hyperpnea. Anoxic blood produces reflex hyperpnea in 100 per cent of experiments over the whole range of 4 to 20 vols. per cent decrease in O_2.

As figure 9 shows, the carotid body responded in each of 44 observations to reduction in O_2 content of the blood amounting to 4 vols. per cent or more. To increased CO_2 there was no reflex response in any of 8 cases below 4 vols. per cent, a response in half of 12 cases at an increase ranging from 4 to 8 vols. per cent, in 55 per cent of 9 cases with increases between 8 and 12 vols. per cent, and in 77 per cent of 9 cases over the range of 12 to 16 vols. per cent. It was only when the increase amounted to more than 16 vols. per cent that the response was constant; the number of the latter observations is too small to permit definite conclusions, but the general tendency is unmistakable.

These results indicate that anoxemia is a more consistently effective stimulus than hypercapnia to the carotid body receptors. They also suggest that over the usual physiological range anoxemia is the more powerful stimulus. Additional evidence in that direction will be presented below.

B. *Relation of the intensity of the systemic response to the number of reflex units in operation.* In all these experiments, only one carotid body was perfused at a time, so that reflex responses were necessarily elicited from only one set of receptors. However, during the systemic responses produced by inhalation of CO_2, N_2, or N_2O, either one, or both, or neither carotid body might be functioning. Thus in group A (fig. 10) neither carotid body was functioning during CO_2 or N_2O inhalations (one carotid body excluded by denervation, the other excluded by perfusion during the inhalation at a pressure above systemic blood pressure). In group B only one carotid body was receiving systemic blood (the other either denervated or being perfused). In group C both carotid bodies figure in the systemic response to CO_2 and N_2O (gases administered before perfusion was begun; carotid body innervation intact bilaterally). This grouping enabled us to correlate the intensity of the hyperpnea elicited by the inhalation of the gases with the number of reflex units which participated in the systemic response, since the depressor nerves were cut in all cases. We could also correlate the intensity of the hyperpnea elicited by the inhalation with that produced reflexly by perfusion of the same blood through one carotid body. The results of such correlations are shown in figure 10.

They confirm those previously obtained by Heymans et al., by Schmidt, and by other observers in indicating that the hyperpnea produced by inhalation of CO_2 is not significantly affected by inactivation of the carotid chemical receptors, for the average systemic responses were practically the same whether one carotid body, or both, or neither participated in the effect of the inhalation. With anoxemia (again confirming previous work) there was practically no hyperpnea at the time of the inhalation if neither carotid body was functioning (group A); if one of the bodies was functioning the inhalation produced a hyperpnea which, on averaging the individual observations, turns out to be almost exactly equal to that elicited by perfusion of the same blood through the other carotid body (group B) when both bodies participated in the systemic response the average hyperpnea was approximately twice as great as that elicited by perfusion of one body (group C). The individual observations are not shown in this chart (see fig. 11, which is constructed from the same data) but there is nothing in them to necessitate modification of the conclusions drawn from their averages.

The reflex hyperpneas of anoxemia are again shown by figure 10 to be

more intense than those produced by hypercapnia, while the systemic responses to hypercapnia are usually more intense than those to anoxemia. There is no sign that carotid reflexes play any significant part in the systemic response to CO_2 while the response to anoxemia is clearly dependent upon them.

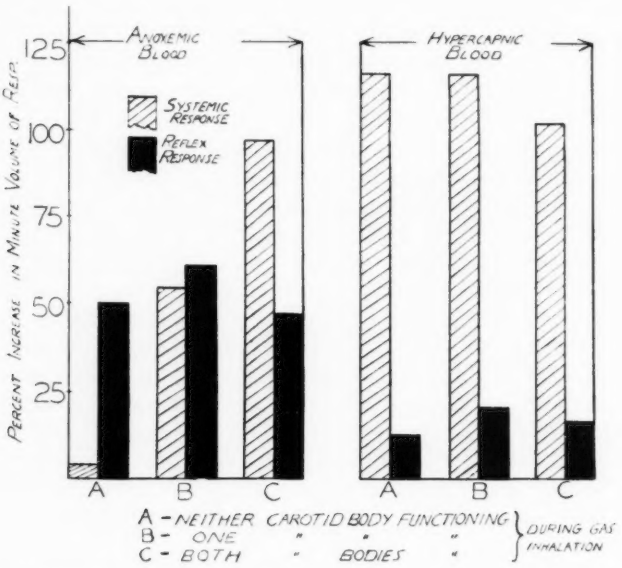


Fig. 10. Relation of intensity of systemic and reflex responses as influenced by the number of reflex units in operation during the inhalation.

This chart is constructed with the same data shown in figures 9 and 11. Each vertical column represents the average of a number of observations regardless of extent of change in gas content of the blood. Never more than one carotid body was perfused at a time (reflex response), though during inhalation of CO_2 or N_2O (systemic response) neither (A), one (B), or both, (C), carotid bodies could be functioning and receiving systemic blood. For each observation in chart on left, there is a consecutive observation on the same animal in the chart on right. Note that the systemic response to anoxemia varies with the number of reflex units in operation, but the systemic response to CO_2-excess is essentially the same regardless of the number of functioning reflex units.

C. *Comparison of the central and reflex hyperpneas produced by a given change in the blood.* The 41 observations made with varying degrees of hypercapnia and the 44 corresponding observations with anoxemia are shown in relation to the change in the blood gas content in figure 11. The changes in the blood are grouped in increments (CO_2) or decrements (O_2) of 2 vols. per cent; the average change in minute volume of breathing for

the group is indicated by the large blocks, the unshaded ones showing the average systemic responses, the black ones the average of the responses elicited by perfusing the same blood through one carotid body. The individual observations of respiration are also indicated, systemic effects as small squares, reflex effects as small circles; the numbers within the squares and circles in the same group indicate corresponding observations, i.e., figure in square and figure in circle show the systemic and reflex responses elicited by the same blood in the same animal.

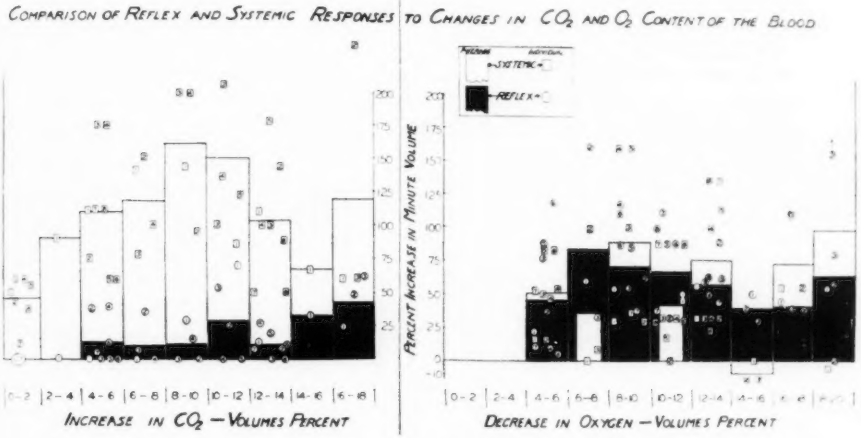

COMPARISON OF REFLEX AND SYSTEMIC RESPONSES TO CHANGES IN CO_2 AND O_2 CONTENT OF THE BLOOD
PERCENT INCREASE IN MINUTE VOLUME
INCREASE IN CO_2 — VOLUMES PERCENT
DECREASE IN OXYGEN — VOLUMES PERCENT

Fig. 11. Constructed from the same data as figures 9 and 10, but individual observations are shown, grouped in increments of 2 vols. per cent for CO_2 and decrements of 2 vols. per cent for O_2. The unshaded and black columns represent *average* systemic, and *average* reflex responses respectively; the small numbered squares and circles represent *individual* systemic and *individual* reflex responses respectively. These latter are so arranged that the numbers correspond for each column, so that figure in square and figure in circle represent systemic and reflex response of an animal to the same blood. Number of reflex units in operation during the systemic response is not considered here (see fig. 10).

The CO_2 chart shows clearly that only a moderate increase in CO_2 content of the blood is sufficient to cause a definite hyperpnea systemically, but insufficient to stimulate the carotid body receptors. Increase in CO_2 up to 2 vols. per cent systemically increased respiration 50 per cent (average of 7 experiments); increase from 2 to 4 vols. per cent practically doubled respiration (one observation), but in none of these 8 observations was the same blood adequate to stimulate the carotid body when perfused through it. Only when the increase in blood CO_2 exceeded 4 vols. per cent did reflex hyperpneas begin to appear, and even then they were inconstant (compare fig. 9). The systemic response increased progressively up to

an increase of 10 vols. per cent in blood CO_2 and then declined; this may have been due to the narcotic effects of high concentrations of CO_2. The reflex hyperpnea shows a definite tendency to increase in intensity when blood CO_2 was increased more than 10 vols. per cent, but reflex hyperpneas were not seen constantly until the increase in CO_2 amounted to more than 14 vols. per cent (compare fig. 9). The intensity of the reflex hyperpnea was with only one exception decidedly less than that elicited by the inhalation; the exception lies in the 16 to 18 vols. per cent column (observation 3—actual increase in CO_2 17.1 vols. per cent), the reflex hyperpnea equalling the systemic with this extreme rise in blood CO_2.

Beginning with a reduction of 4 vols. per cent in blood oxygen reflex hyperpnea occurred without exception in all of the 44 observations on 14 animals. The intensity of the reflex response increased slightly when the anoxia reached 6 to 8 vols. per cent, but it did not increase any further when the reduction exceeded 8 vols. per cent. The relation of the systemic to the reflex response is quite irregular, depending upon the preponderance in the group of animals having both carotid bodies, or one, or neither in action during the inhalation (compare fig. 10); thus in the 6–8 and 10–12 anoxemia columns the average reflex hyperpnea exceeded the average systemic and in the 14–16 column there was no hyperpnea during the inhalation in any of the 3 cases; since depression of breathing occurred in 2 of them, the average systemic response is negative in this group.

D. *Other observations.* a. *The influence of changes in hydrion concentration.* In 17 of the observations with anoxia and in 37 of those with hypercapnia pH determinations were made on the blood samples by means of the Beckman glass electrode apparatus. In the anoxic bloods pH was invariably increased, the increase ranging from 0.07 to 0.23; 9 of the 17 showed pH increases of 0.11 to 0.20, and two showed rises of 0.22 and 0.23; three of the remainder showed an increase of 0.1. In the CO_2 bloods there was a fall in pH in all but one, in which the CO_2 content was increased only 0.5 vol. per cent and pH was unchanged. In the others pH fell by 0.09 to 0.33, and in 15 of them the fall amounted to 0.2 or more; in only 6 was the fall less than 0.1, and in 15 it lay between 0.1 and 0.2. Since the anoxic bloods caused a consistent and marked reflex hyperpnea although the blood was more alkaline and the hypercapnic bloods were less effective in arousing reflexes although the blood was more acid, it is evident that changes in hydrion concentration per se have no significant effects upon the carotid body receptors over the physiological range studied in these experiments.

Additional evidence pointing in the same direction was obtained in four experiments in which part of a blood sample collected during oxygen inhalation was treated with acid (3–5 cc. N/10 HCl per 100 cc.) and perfused through the carotid body in alternation with the untreated part of the

same sample. The fall in pH ranged from 0.2 to 1.0, but in no case did alternation of acidified with control blood alter respiration in the slightest. Yet in each experiment the carotid body was unusually sensitive to lobeline and anoxemia both before and after perfusion with acidified blood.

A typical experiment was as follows: 150 cc. of blood were withdrawn during oxygen inhalation. To 70 cc. of it, 3.5 cc. of N/10 HCl were added; pH of the untreated part was 7.1, that of the acidified part 6.1; the corresponding figures for CO_2 content were 36.1 and 25.6 vols. per cent. Respiration showed no change whatever when the two parts of the sample were interchanged in the perfusion system although the change in pH was 1.0. This animal was very reactive to lobeline and to anoxic blood, both before and after the perfusion with acidified blood.

b. *The threshold of the carotid body receptors to changes in CO_2 tension.* The smallest rise in blood CO_2 by which a reflex hyperpnea was elicited was 4.7 vols. per cent; pH was reduced by 0.22. This represents an increase in CO_2 tension of approximately 20 mm. of mercury, which is the smallest increase by which measurable stimulation of the carotid body receptors was produced in these experiments. The reflex hyperpnea in this case was a 40 per cent increase in respiratory minute volume while the increase during the inhalation was 175 per cent. No attempts were made to estimate the oxygen tension in the blood samples.

c. *The effect of changes in perfusion pressure.* In nearly every experiment we had opportunities to observe the behavior of respiration and circulation while the pressure in the perfusion system was altered without altering the chemical composition of the fluid. There were never any significant changes in respiration or in blood pressure even when perfusion pressure was altered by more than 200 mm. of mercury; no observations were made of pulse rate at the time but the records show no evidence of changes. Apparently there are no pressure receptors in the carotid body of the dog.

d. *The effect of reflexes from the chemical receptors upon the circulation.* The changes in respiratory activity produced by alterations in the chemical composition of the blood perfusing the carotid body were not accompanied by any striking or consistent changes in blood pressure. The changes that did occur were slight and might well have been secondary to the altered activity of respiration; there was no definite evidence that the vasomotor center was markedly affected by the reflexes aroused by changes in the CO_2 or oxygen content of the blood. Injection of lobeline into the perfusion stream frequently caused an instantaneous but very fleeting inhibition of the heart if the vagi were intact and occasionally it led to a rise in blood pressure, but similar results were never obtained with anoxic or hypercapnic blood. Perhaps if the latter chemical excitants had been introduced into the carotid body as a sudden overwhelming wave—as was the case with lobeline—similar circulatory effects might have been elicited

by them. This was not possible with our perfusion system for blood of altered gas content mixed with that previously used before displacing it.

DISCUSSION. From these experiments it seems quite clear that the chemical receptors of the carotid of the dog are located entirely in the carotid body, as de Castro and Heymans have claimed. This unusual specialization of the "chromaffine" tissue of the carotid body has obvious implications to the function of similar tissues elsewhere; further investigations are now under way. It is also quite clear that in the dog, under the conditions of these experiments, anoxemia is a more powerful stimulus than hypercapnia to the carotid body receptors whereas hypercapnia is definitely the more powerful stimulus to the respiratory center. Since an increase in blood CO_2 sufficient to double the volume of respiration by central action was insufficient to stimulate the carotid bodies of these animals, the center is obviously much more sensitive than the reflex system to CO_2. We believe that this reflex mechanism is brought into play only by unusual emergencies such as foreign chemicals (cyanide, sulphide, nicotine, lobeline), anoxemia, and marked increases in the CO_2 tension of the blood, while the normal control of breathing is accomplished mainly by the direct effects of CO_2 upon the center. The reflex receptors seem to be specialized to respond to changes in the oxygen tension rather than the CO_2 tension of the blood while the cells of the center are specialized to respond to the latter rather than the former. We suggest that the carotid body receptors are essentially structures in which an interference with oxidations gives rise to afferent nerve impulses stimulant to the respiratory center. This is based on the intensity of the reflex hyperpnea elicited by anoxemia and on the similar effects of cyanide and sulphide, which are characteristic inhibitors of tissue oxidations. Nicotine, another stimulant to the receptors, inhibits oxidations in some living systems; lobeline apparently has not been tested in regard to biological oxidations.[4]

[4] The carotid body receptors are also stimulated by choline and certain of its derivatives. Dautrebande and Marechal (Compt. Rend., 1933, *113*, 76) found that carbaminoyl choline ("Doryl") caused immediate hyperpnea when injected into the carotid of the dog, and Heymans (Compt. Rend., 1935, *120*, 1350) obtained similar results with acetyl choline. We have confirmed these observations and have also found that choline itself and the methyl ether of β-butyl choline (which has powerful nicotinic actions with scarcely any muscarinic potency) act similarly. All these substances cause reflex hyperpnea after full atropinization: the action is therefore nicotinic, not muscarinic. This is further borne out by observations with choline derivatives which have little or no nicotinic potency (the acetyl and carbamic esters and the ethyl ether of β-methyl choline), for these failed to cause any carotid body stimulation.

The carotid body receptors are therefore *cholinergic* in the nicotinic sense. This fact may be interpreted in various ways. We are inclined to believe that interference with oxidations in the receptors is the responsible factor in the case of these substances as well as cyanide, sulphide, nicotine, and lobeline. Direct evidence is not available at present.

The effects of high tensions of CO_2 may also be explained on this basis as the result of interference with oxidations in the receptors because of interference with the removal of CO_2 produced by them.

One of the most remarkable features of this reflex mechanism is its ruggedness. It is only stimulated by anoxia of all degrees and even enormous increases in CO_2 tension do not appreciably depress it, whereas

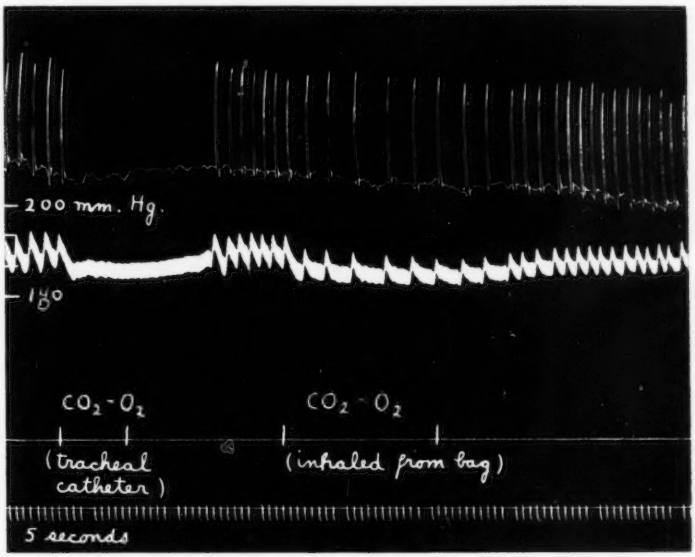


Fig. 12. Respiratory depression following administration of CO_2-O_2 in dog with severe depression of respiratory center.

Dog. Respiration markedly depressed following repeated intravenous injections of chloralose. Transient stimulation of respiration had occurred after lobeline intravenously but when a gentle stream of 10 per cent CO_2 in O_2 was introduced directly into the lungs by a tracheal catheter, respiration ceased completely for 2 minutes. Less intense administration of CO_2-O_2 (inhaled from a bag attached to the tracheal cannula) again depressed respiration, though to a lesser extent. Note that the CO_2 of the mixture failed entirely to stimulate breathing; the result was exactly the same as that produced by pure O_2. The respiratory center evidently was kept active only by afferent impulses aroused by anoxemia.

the cells of the center are depressed both by severe anoxia and by excessive hypercapnia. In animals given large amounts of depressant drugs breathing often is maintained by reflexes from the carotid bodies and aorta after the center has lost practically all of its ability to respond to CO_2. Removal of anoxemia by means of insufflation or inhalation of oxygen then causes depression or cessation of breathing, and addition of CO_2 to the inspired

oxygen makes no difference (fig. 12). Under similar circumstances removal of the reflexes by section of the depressor and sinus nerves causes respiratory failure. Thus the chemical reflex mechanism appears to be the *ultimum moriens* of the respiratory regulating system; it continues to furnish afferent impulses which excite the cells of the center when they are so depressed that they have lost their specialized capacity of responding to changes in their immediate environment.

It is remarkable that reflexes from the carotid bodies should constitute an essential and apparently very primitive factor in the body's defense against anoxemia and other agents which depress nerve cell function. A possible explanation lies in the embryology of the carotid reflex system. Koch (1931) pointed out that the parts of the circulation in which specialized pressure receptors are located are adult survivals of the primitive branchial arterial system of the embryo; the carotid sinuses correspond with the third branchial arch arteries, and the aortic arch with the fourth branchial arch artery on the left side. The development of a reflex system reacting to pressure changes thus seems to be related to survival of a primitive organization found in the gill arteries of the embryo. The carotid body, according to Boyd (1937), develops from the same mesodermal cells which give rise to the third branchial artery, but the former receives nerve elements from the glossopharyngeal at an earlier stage and this apparently determines its subsequent differentiation. Koch's explanation for the location of the pressure receptor system could therefore be applied to the chemical reflex system as well. For reasons as yet unknown, the latter is responsive to chemical agents and is most directly concerned with the respiration, while the former responds to pressure changes and is most concerned with the circulation. Both sets of receptors are conspicuously resistant to adverse conditions, and this may be because they represent primitive embryonal structures.

Although the conditions of these experiments were necessarily abnormal in certain respects, we believe that the conclusions drawn from them are applicable to the intact animal. The narcotic used by us—chloralose—can be shown in decerebrate dogs to decrease the sensitivity of the respiratory center to CO_2 and at the same time to increase the effectiveness of carotid sinus reflexes; under its influence the balance of factors in respiratory control is shifted away from the central and toward the reflex. If then CO_2 still shows a preponderance of central over reflex factors—as it did in our experiments—this should be even more marked in the intact animal. The abnormalities due to dissection, perfusion, and the use of only one carotid body at a time, were equally operative for anoxemia and hypercapnia; they do not interfere with the conclusion that anoxemia is the more consistent and more powerful stimulus. We believe that the relative parts played in the chemical control of breathing by reflexes and by direct

effects on the center are shown quite accurately by comparing the respiratory effects of hypercapnia and anoxemia. The sensitivity of the intact organism to hypercapnia is much greater than that to anoxemia and the intensity of the response to the former is usually much greater than that to the latter. The response to anoxemia represents maximal participation of the reflex factor with little or no central component while that to hypercapnia represents direct effects on the cells of the center with little or no reflex component. The reflex mechanism is the less sensitive and produces the smaller effect.

Our observations with carbon monoxide, although few, deserve a brief comment. It has been known since the work of Haldane (1922) that monoxide does not elicit hyperpnea in proportion to the anoxia produced by it and this has been attributed to the fact that in CO poisoning the oxygen tension of the arterial blood remains normal as long as breathing is adequate. This indicates that the carotid body receptors are satisfied with a small amount of oxygen provided that the tension remains normal. The carotid body receives arterial blood directly from the carotid system without passage through or admixture with blood from other active tissues. The amounts of oxygen carried in simple solution in the blood evidently are more than adequate to meet the needs of the small mass of tissue which the receptors represent, but the tension of the gas must be maintained nearly at a normal level for this to occur. Hence the reflex hyperpnea produced when the carotid body was exposed to blood equilibrated against monoxide ceased as soon as the blood was again permitted to take up oxygen, even though the amount of CO combined with the blood was not yet measurably reduced. Monoxide evidently does not inactivate the receptors—which might account for the absence of marked hyperpnea from this gas—because we found reflex hyperpnea to occur when blood deoxygenated by monoxide was perfused through the carotid body and after perfusion with monoxide-containing blood the receptors were normally responsive to lobeline and to anoxic blood.

SUMMARY AND CONCLUSIONS

1. The nerve receptors responsible for the hyperpnea produced by intracarotid injections of lobeline and cyanide are located, in the dog, entirely in the carotid body. The receptors of the carotid sinus are insensitive to these agents and the receptors of the carotid body are insensitive to pressure changes.

2. The carotid body is stimulated either by reduction in the oxygen content or increase in the CO_2 content of the fluid perfusing it; the reflex hyperpnea is consistently greater with anoxic than with hypercapnic fluid, and the degree of hypercapnia required is usually greater than the degree of anoxemia. Carbon monoxide causes reflex hyperpnea when the

blood is deoxygenated by it, but the hyperpnea disappears as soon as the blood is permitted to take up oxygen and before its CO content is measurably reduced.

3. The physiological significance of carotid body reflexes was studied in experiments in which the animal (dog) was used as a tonometer and the systemic and reflex effects of the same blood could be compared; the following conclusions were reached:

a. Carotid body reflexes constitute an accessory mechanism, brought into action by emergencies such as foreign chemicals, anoxemia, and unusually great increases in the CO_2 tension of the blood, rather than an essential part of the normal respiratory regulating system; the control of breathing under ordinary conditions is accomplished entirely by the direct effects of chemical stimuli (mainly CO_2) upon the cells of the center.

b. The carotid body receptors are specialized to respond to interference with oxidations within them, hence their reactions to cyanide, sulphide, nicotine, and anoxemia; excess CO_2 may act similarly. The cells of the center are of course specialized to respond to changes in CO_2 tension and they normally are much more sensitive to this than the carotid body receptors are to anoxia; the central factor is also more powerful than the reflex in producing changes in pulmonary ventilation.

c. The carotid body receptors are more resistant than the cells of the center to depression by narcotics, anoxia, and excessive CO_2 tension; this reflex mechanism is the *ultimum moriens* of the respiratory regulating system and it frequently keeps respiration going when the cells of the center have lost practically all of their ability to respond to changes in their immediate environment. A possible reason for this, based on the embryology of the carotid body is suggested.

4. Reasons are presented for believing that these deductions concerning the relative importance of central and reflex factors are applicable to the control of breathing in the normal animal.

REFERENCES

ADDISON, W. H. F. AND J. H. COMROE, JR. Anat. Record **67:** supp. 3, p. 7, 1937.

BOGUE, J. Y. AND G. STELLA. J. Physiol. **83:** 459, 1935.

BOUCKAERT, J. J., L. DAUTREBANDE AND C. HEYMANS. Ann. de Physiol. **7:** 207 1931.

BOYD, J. D. Pub. no. 479 Carnegie Instit. of Wash., pages 1–32, Jan. 30, 1937.

DE CASTRO, F. Trav. Labor. Rech. Biol. Univ. Madrid **24:** 365, 1926; **25:** 331, 1928.

GAYET, R., D. BENNATI AND D. QUIVY. Arch. Internat. de Pharmacodyn et de Therap. **50:** 129, 1935.

GEMMILL, C. L. AND D. L. REEVES. This Journal **105:** 487, 1933.

GOLLWITZER-MEIER, K. AND H. SCHULTE. Pflüger's Arch. **229:** 251, 1931.

GOLLWITZER-MEIER, K. Pflüger's Arch. **234:** 342, 1934.

GREEN, M. F. AND A. DEGROAT. This Journal **112:** 488, 1935.

HALDANE, J. S. Respiration. Yale Univ. Press, 1922.

HEYMANS, C., J. J. BOUCKAERT AND L. DAUTREBANDE. Arch. Internat. de Pharmacodyn et de Therap. **39:** 400, 1930.
HEYMANS, C. AND J. J. BOUCKAERT. Compt. Rend. Soc. de Biol. **112:** 1240, 1933.
HEYMANS, C., J. J. BOUCKAERT AND P. REGNIERS. Le Sinus Carotidien. G. Doin, Paris, 1933.
HEYMANS, C. AND P. RIJLANT. Compt. Rend. Soc. de Biol. **113:** 69, 1933.
KOCH, E. Die Reflektorische Selbststeuerung des Kreislaufes. Steinkopf, Dresden, p. 7, 1931.
MIES, H. Ztschr. f. d. ges. Exper. Med. **85:** 282, 1932.
MULINOS, M. G. J. Pharmacol. Exper. Therap. **51:** 135, 1934.
OWEN, H. AND R. GESELL. Proc. Soc. Exper. Biol. and Med. **28:** 765, 1931.
SAMAAN, A. AND G. STELLA. J. Physiol. **85:** 309, 1935.
SCHMIDT, C. F. This Journal **102:** 94, 1932.
SELLADURAI, S. AND S. WRIGHT. Quart. J. Exper. Physiol. **22:** 233, 1932.
SMYTH, D. H. J. Physiol. **88:** 425, 1937.
VAN SLYKE, D. D. AND J. M. NEILL. J. Biol. Chem. **61:** 523, 1924.
VON EULER, U. S. AND G. LILJESTRAND. Skand. Arch. f. Physiol. **74:** 101, 1936.
WINDER, C. V. This Journal **118:** 389, 1937.
ZOTTERMAN, Y. Skand. Arch. f. Physiol. **72:** 73, 1935.

TOTAL URINARY ESTROGEN, ESTRONE AND ESTRIOL DURING A MENSTRUAL CYCLE AND A PREGNANCY

GEORGE VAN S. SMITH, O. WATKINS SMITH AND GREGORY PINCUS

From the Fearing Research Laboratory, Free Hospital for Women, Brookline, Mass.,[1] *and the Biological Laboratories, Harvard University*[2]

Received for publication July 12, 1937

The quantitative determination of the estrogenic potency of human urine under various physiological and pathological conditions has been the object of considerable study with widely diversified results. One aim of this investigation was to compare values acquired by the use of two different procedures. Since a very large proportion of the urinary estrin can be obtained only after hydrolysis to free it from the conjugated state in which it is excreted, we have analyzed urines during a menstrual cycle and a pregnancy comparing two methods of acid hydrolysis which seem to give maximum yields, namely, that of Smith and Smith (1) and that of Cohen and Marrian (2).

A second aim was to separate the estrin extracted by the latter method into estrone and estriol fractions. Inasmuch as Pincus and Zahl (3) have shown that progestin, in addition to causing increased excretion of injected estrin in rabbits (as originally reported by Smith and Smith, 4) also augments the conversion of estrone to estriol, it seemed possible that in humans also the presence of progestin might be characterized by increased excretion of estriol, and that a study of estrone and estriol in the urine might give some quantitative index of progestin, both in the menstrual cycle and in pregnancy.

Finally, the determination of urinary estrone and estriol throughout a menstrual cycle and a pregnancy has not, to our knowledge, been previously reported.[3]

METHODS. The Smith and Smith method was employed as previously described, (1) except for the introduction of a reflux condensor during the 10-minute period of acid hydrolysis. The Cohen and Marrian method of hydrolysis and separation was followed, using the slight modifications described elsewhere (3). All extracts were

[1] The Mrs. William Lowell Putnam Investigation of the Toxemias of Pregnancy.

[2] Aided by a grant from the Josiah Macy, Jr., Foundation.

[3] Cohen et al. (5) published a graph for estrone and estriol during pregnancy but this was based on the average values of 80 specimens from 20 different women at various stages of gestation.

titred by bioassay. By adding crystalline estrone and estriol to a non-pregnancy urine of known potency we found, by bioassay, that these two substances were quantitatively recovered by the Smith and Smith and the Cohen and Marrian methods, thus precluding any possibility of destruction or conversion of these two compounds during hydrolysis. The separation of estrone and estriol added to a non-pregnancy urine was also shown to be quantitative by the Cohen and Marrian procedure.

Colorimetric determinations of the fractionated materials were also performed, but with the fractions from the urines of the menstrual cycle very large overestimates were obtained, due to the presence of inactive chromogenic material. This chromogen was associated with a red pigment found most concentrated in the estriol fraction. In urines collected after the second month of pregnancy the estrone fractions were either completely or practically completely free of this pigment. The estriol fractions of specimens from the sixth month on contained only small amounts of it and the colorimetric determinations did not give large overestimates on comparison with values derived by bioassay (6).

In performing bioassay we use mature spayed female rats. All animals are frequently standardized for their reactivity against an estrogen of known potency, and each test animal is put into estrus, either with an unknown or the standard preparation, once every week. Smears are taken just prior to injections to be sure the diestrous state has been reëstablished. All injections, both for priming the animals and for testing unknowns, are given in three divided doses 4 hours apart and the results based on vaginal smears taken 48 hours from the first injection. Pre-estrous, full estrous or post-estrous smears are considered positive. The smallest amount of material which gives positive smears in at least 4 out of 6 rats is considered one rat unit, provided no more than 2 out of 6 rats give positive smears when injected with the next smaller dosage.

By this method of assay 1 microgram of crystalline estrone is equivalent to 2.0 rat units, 1 microgram of estriol to 2.5 rat units, and 1 microgram of estradiol to 20 rat units. The high value for estriol is probably accountable to the fact that a pre-estrous smear is considered positive by our criterion. Estrone and estradiol also give high titres, possibly because of the fact that our animals are kept in a constant state of reactivity.

All results are expressed in terms of rat units per 24-hour volume of urine. The potency of the estrone and estriol fractions might be converted into terms of micrograms, but this has been omitted in the charts in order to be able to compare these values with those for "total" estrin acquired by the Smith and Smith method, in which no separation is performed.

"Total" estrogens during a menstrual cycle. (Chart 1.) This woman, aged 27, had kept a calendar record of her catamenia during the previous year and had had 27- to 29-day cycles. From January 12 through February 8, 1937, all of her urine was saved, 48-hour rather than 24-hour amounts being taken in order to have enough material for both methods. The 13 specimens were extracted within 4 days of the time of collection. In 5 instances the sum of estrone plus estriol gave values for "total" estrin which were significantly higher than those acquired by the Smith and Smith method. In 2 instances the Smith and Smith method gave significantly higher values. With the other 6 specimens the values for "total" estrin by the two methods checked within the limits of the errors

involved in biological assay. The shapes of the two curves are essentially similar except that the Cohen and Marrian is higher during the second half of the cycle.

Inasmuch as the estrogenic potency of non-pregnancy urine is too low to permit the assay of unextracted specimens after acid hydrolysis, we were unable to discover whether the difference in results was due to difference of hydrolysis or extraction. The methods employed compare favorably with others as to total recovery. Thus, Gustavson et al. (7) obtained activity equivalent to a maximum total excretion of

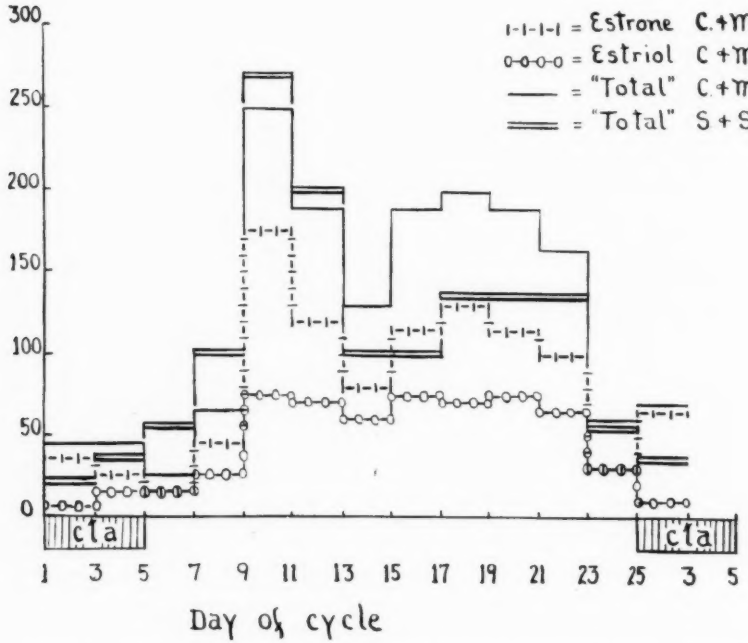


Chart 1. "Total" estrin, estrone and estriol during a menstrual cycle. Rat units in 24 hours.

1.3 mgm. of estrone per cycle, and we obtain 0.99 mgm. of estrone plus 0.46 mgm. estriol; in terms of estrone equivalent this woman excreted 1.67 mgm. during this cycle. The maximum daily excretion reported by Gustavson et al. is 90 micrograms (in terms of estrone equivalent). Maximum excretion in this 25-day cycle came on the fourteenth to sixteenth day before menstruation, when 87 micrograms of estrone and 30 micrograms of estriol per 24 hours were excreted, making a total of 126 micrograms per 24 hours in terms of estrone equivalent. The maximum excretion came about two days earlier in this cycle than in those previously reported by us (8). There was also a secondary rise during the last half of the cycle, as has been found by Gustavson et al. (7). We have not previously encountered this phenomenon.

Estrone and estriol during a menstrual cycle. (Chart 1.) Estrone-injected rabbits having corpora lutea and uteri excrete more estriol than estrone (3). From the work of MacCorquodale et al. (9), however, it would appear that the ovary's primary estrogen is estradiol. It has recently been shown (10) that estradiol, upon injection into rabbits, is converted, when the ovaries are present, into estrone and excreted as estrone and estriol. Even in the presence of progestin a predominance of estrone maintains, due to the fact that the rate of conversion of estrone to estriol does not keep pace with the rate of excretion of both.

The results tabulated are in keeping with the assumption that in the human also the ovarian product, estradiol, is converted into estrone and that the degree of conversion of estrone into estriol depends upon progestin. More estrone than estriol was excreted throughout the period of study, as is the case in an estradiol injected rabbit. Estriol, however, maintained a comparatively high level during what was presumably the luteal phase of the cycle, in this case the ninth to the twenty-third day. A drop in estriol preceded the onset of menstruation and low levels were found during the first 9 days, the period of follicle ripening. A drop in estrone was also noted in the specimen which covered the 48 hours before bleeding began. These findings support both the progestin-withdrawal and the estrin-withdrawal theories of menstruation. With the beginning of flow, however, there was a decided increase in the potency of the estrone fraction, and the ratios of estrone to estriol during the first two days of both catamenia were twice as high as those found at any other time in the cycle, suggesting that something more than a withdrawal phenomenon may be concerned in the initiation of bleeding.

Total estrogens during a pregnancy. (Chart 2.) This patient, Mrs. Y. B., aged 25, was followed from the seventeenth day before her last catamenia to the end of gestation. The results for "total" urinary estrin (Smith and Smith method) to the fourth missed period, for F.S.H. prior to conception and for A.P.L. after conception have been published and discussed (8). From the forty-ninth day of pregnancy to delivery urinary estrogens were determined by both the Cohen and Marrian and the Smith and Smith methods. The pregnancy was clinically normal except for a few days of flow at the second missed period and false labor two weeks before delivery.

Of the 18 specimens analyzed by both techniques 11 gave significantly higher values by the Smith and Smith than by the Cohen and Marrian. With the other 7 the values checked within the limits of the error involved in biological assay. In an attempt to discover whether the greater recovery by the Smith and Smith method was accountable to hydrolysis or extraction, 7 different samples of pregnancy urine were studied. The results are presented in table 1. In every instance the two procedures of

hydrolysis, when the assay was made on unextracted specimens, yielded check values. Benzene extraction gave, without exception, a quantitative recovery of the estrogens free after acid hydrolysis. In two of the 7 specimens there was a significant loss of potency (30 to 50 per cent) by the Cohen and Marrian extraction. Any difference between the results for "total" estrin during pregnancy, therefore, would appear to be account-

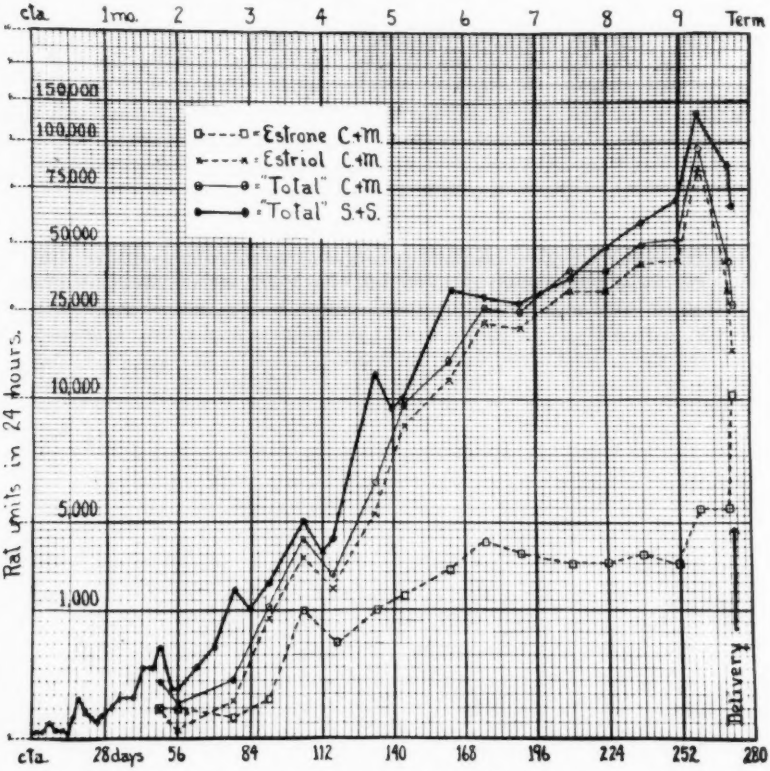


Chart 2. "Total" estrin, estrone and estriol during a pregnancy. Rat units in 24 hours.

able to the chance for loss in the Cohen and Marrian extraction and separation rather than to the technique of hydrolysis.

The total recovery of estrin compares favorably with that reported by Cohen et al. (5). On the basis of colorimetric determination they obtained a maximum average yield (two weeks before delivery) of 22.2 mgm. estriol and 3.0 mgm. estrone per 24 hours. The maximum (at 9 months and 8 days) in these data amounts to 35.2 mgm. estriol and 2.9 mgm. estrone.

The two procedures gave curves for "total" estrin which are essentially similar, the only variation being a cyclic increase of excretion by the Smith and Smith method with peaks every 28 days up to the fifth month. These peaks were not found by the Cohen and Marrian method and, considering the infrequency of specimens after the second month and the errors involved in biological assay, may well be more apparent than real. The most striking feature of the curves for "total" estrin, demonstrated by both methods employed, is the marked drop immediately preceding labor and delivery. This observation confirms the findings of other workers (5) (11).

TABLE 1

Comparison of results of the Cohen and Marrian and the Smith and Smith methods of hydrolysis and extraction. "Total" estrin in pregnancy urines. Rat units in 24 hours

CASE	DATE	STAGE OF PREGNANCY	S. AND S. HYDROLYSIS		C. AND M. HYDROLYSIS		
			Unextracted	Benzene extract	Unextracted	Benzene extract	C. and M. ether extract (estrone + estriol)
		months					
L. D.	November 5, 1935	5	22,000	20,000	20,000	21,000	14,400
F. L.	December 21, 1935	4	17,000	21,000	18,500		17,900
Y. B.	July 18, 1936	4½	15,800	14,400	12,800	11,600	6,300
Y. B.	September 23, 1936	6½	28,000	30,000	22,500		25,300
Y. B.	October 14, 1936	7	38,000	40,000	43,000		41,700
Y. B.	October 28, 1936	7½	48,000	50,000	40,000		43,000
Y. B.	November 11, 1936	8	60,000	58,000	47,000		50,500

Marrian et al. (12) lay stress upon an increase in "free" unconjugated estrins at the time of delivery. We also have made this latter observation, but, inasmuch as urine excreted during labor is usually highly concentrated and acid and since the degree of conjugation probably depends mainly upon its physical and chemical properties, (1) we attach little physiological significance to values acquired on unhydrolyzed specimens.

Estrone and estriol during pregnancy. (Chart 2.) The separation of the two urinary estrogens revealed that the ascendancy of estriol over estrone did not begin until after the second missed period. Moreover, at the time of the second missed period, when the patient had been put to bed because of flowing, three times as much estrone as estriol was being excreted. This rise in the estrone-to-estriol ratio was accountable to a drop in estriol, suggesting a deficiency of progestin. The fact that it coincided with flowing is of especial interest in connection with the high estrone-to-estriol ratio observed at the onset of menstruation (chart 1).

From about the seventieth day on the rise in total estrogen was due to

an increased excretion of both estrone and estriol, the latter compound being in the ascendancy throughout. The ratio of estriol to estrone steadily advanced from 2 (at 2 months and 21 days) to 15 (at 9 months and 8 days). If one may assume that in the pregnant woman progestin facilitates the conversion of estrone to estriol, these data give evidence for progestin in increasing amounts as pregnancy progresses. The probable source of this factor is in the placenta and the fact that 15 times as much estriol as estrone was excreted in late pregnancy, whereas in the non-pregnant woman estrone predominated even during the luteal phase of the cycle, suggests that the human placenta is capable of producing a great deal more progestin than is the ovary.

Twelve days prior to delivery the patient went into false labor. At this time a rise in estrone as well as estriol was noted, and the "total" estrogen was nearly twice as high as it had been a week earlier. The estriol-to-estrone ratio, however, had not changed, indicating that there had been no change in the amount of progestin. The pains subsided and the next specimen covered the 12 hours before true labor began. The estrone fraction of this urine showed the same potency as that of the preceding specimen, but a marked drop in estriol resulted in a lowering of the estriol-to-estrone ratio from 15 to 6.6. The last specimen covered the time of labor and delivery. Separation revealed a further drop in estriol and a marked rise in the potency of the estrone fraction, so that this substance was at its peak value and the estriol-to-estrone ratio was only 1.7, lower than it had been at any time since the second missed period. These findings possibly reflect a decrease in the amount of progestin just prior to the onset of labor and delivery. The marked increase in the amount of estrone (or some estrogen present in the estrone fraction) in the last specimen suggests that in the initiation of labor, as in the initiation of endometrial bleeding, progestin withdrawal may be followed by a positive precipitating factor.

The composite curve of Cohen et al. (5) shows a drop in both the estrone and estriol fractions at parturition, although the relative drop in estrone is less than in estriol. Our observations reveal a decided increase in the potency of the estrone fraction. This difference, as will be pointed out below, may be because we use bioassay, whereas their results are based on colorimetric determinations.

Evidence for a third estrogen in the urine of pregnancy. Up to this point we have assumed that the only estrogens present in urine are estrone and estriol. Although this assumption has been generally accepted, comparison of colorimetric with bioassays upon the samples collected after the 112th day of pregnancy gave evidence for activity which could not be accounted for by these two compounds alone. The estrone fractions, after the second month, were practically free of the inactive chromogenic material which reacts with the color reagent, so that the colorimetric values should have been accurate. Contrary to expectation, all of these values after the 112th day were *less* than those determined by bioassay, accounting for only about 70 per cent of the estrogenic potency of the estrone fractions during the fourth and fifth

months and only 20 to 30 per cent at the time of parturition. These differences are great enough to indicate definitely the presence, in the estrone fractions, of some estrogen other than estrone—either one that does not react with the color reagent or one more active than estrone. Estradiol would be the compound suspected under the latter possibility, since it gives no more color with the reagent used than does estrone but is ten times as potent as estrone when tested by bioassay. Inasmuch as the conversion of estradiol into estrone appears to be a function of the ovary (10) and since the ovary becomes less active later in gestation, the presence of unconverted estradiol in increasing amounts as pregnancy advances might be expected, and might even play a part in the mechanism of delivery. In any event, the discrepancy noted between colorimetric and bioassay seems to explain, although only in part, the difference between our results for estrone at the end of pregnancy and those reported by Cohen et al. (5).

Evidence is accumulating that the term estrin covers a group of factors which differ in their physiological activity upon the female genitalia and pituitary, estradiol being the most potent, estrone next and estriol the least potent of these three (13) (14) (15). It is not unlikely, therefore, that values for "total" estrin may fail to reveal the actual physiological situation. In the data herein reported low levels of "total" estrin were invariably due in large part to a decreased excretion of estriol, whereas estrone, the more active compound in all of its physiological effects, was either unchanged (as at the time of abnormal flow in early pregnancy) or actually increased (as at the onset of menstruation and during labor and delivery). The inference from these observations might be that, although withdrawal of progestin precedes these phenomena (as indicated by decreased excretion of estriol), the actual precipitating factor is a rise in the level of a physiologically more potent estrogen.

SUMMARY. The "total" estrogenic potency of urine collected through a menstrual cycle and a pregnancy has been quantitatively measured by bioassay after the use of two different methods of hydrolysis and extraction.

The two techniques yielded similar curves for "total" estrin throughout the menstrual cycle, the Cohen and Marrian, however, giving significantly higher values during the latter half.

Similar curves for "total" estrin were also found by both methods during a gestation. In 60 per cent of the pregnancy urines, however, the Smith and Smith technique gave significantly higher values than those resulting from the Cohen and Marrian. This difference was demonstrated to be due to the chance for loss in the Cohen and Marrian extraction and separation of estrone and estriol in pregnancy urines.

The separation of estrone and estriol in the menstrual cycle revealed that 1, more estrone than estriol was present throughout; 2, more estriol was excreted during the luteal phase than during menstruation and the period of follicle ripening; 3, a rise in estrone accompanied the onset of

menstruation, and 4, the estrone-to-estriol ratio was twice as high during the first two days of both catamenias followed as at any other time.

The separation of estrone and estriol through pregnancy revealed that 1, the only specimen which contained more activity in the estrone than in the estriol fraction was collected at the time of the second missed catamenia when the patient had been put to bed for flowing; 2, estriol was in the ascendancy for the rest of the pregnancy, its rate of increase being greater than that of estrone to the last month; 3, false labor 12 days before delivery was accompanied by a marked rise in both estrone and estriol, the ratio of the two remaining unchanged; 4, labor and delivery were accompanied by a drop in estriol and a rise in estrone so that the ratio of estriol to estrone was lower than it had been at any time since the second month.

Comparison of colorimetric with bioassay on the pregnancy urines from the fourth month on suggested the presence of some estrogen in addition to estrone and estriol and more active than either, e.g., estradiol.

CONCLUSION

These findings indicate that in humans as in rabbits progestin prevents the destruction of estrin and facilitates the conversion of estrone into the physiologically less active estriol. On this basis they offer evidence in support of the assumption that progestin deficiency pertains just prior to menstruation, abnormal flow early in pregnancy, normal labor and delivery and that a *positive* precipitating factor in these phenomena may be the resultant rise in the more active estrogenic compound, estrone.

REFERENCES

(1) Smith, G. V. and O. W. Smith. This Journal **112:** 340, 1935.
(2) Cohen, S. L. and G. F. Marrian. Biochem. J. **28:** 1603, 1934.
(3) Pincus, G. and P. A. Zahl. J. Gen. Physiol., July, 1937.
(4) Smith, G. V. and O. W. Smith. This Journal **98:** 578, 1931.
(5) Cohen, S. L., G. F. Marrian and M. Watson. Lancet **1:** 674, 1935.
(6) Pincus, G., G. Wheeler, G. Young and P. A. Zahl. J. Biol. Chem. **116:** 253, 1936.
(7) Gustavson, G., E. E. Hays and T. R. Wood. Proc. Am. Soc. Biol. Chem., p. xlii, April, 1937.
(8) Smith, G. V. and O. W. Smith. New England J. Med. **215:** 908, 1936.
(9) MacCorquodale, D. W., S. A. Thayer and E. A. Doisy. J. Biol. Chem. **115:** 435, 1936.
(10) Pincus, G. Cold Spring Harbor Symposia on Quantitative Biology, 5, 1937.
(11) Browne, J. S. L. and E. M. Venning. This Journal **116:** 18, 1936.
(12) Marrian, G. F., S. L. Cohen and M. Watson. J. Biol. Chem. **109:** lix, 1935.
(13) Freed, S. C., T. Garvin and S. Soskin. Proc. Soc. Exper. Biol. and Med. **35:** 409, 1936.
(14) Lacassagne, A. Am. J. Cancer **28:** 735, 1936.
(15) Dorfman, R. I. Proc. Am. Soc. Biol. Chem., p. xxiv, April, 1937.

THE INFLUENCE OF DINITROPHENOL AND VITAMIN B_1 ON THE WEIGHT OF THE KIDNEY OF THE ALBINO RAT

ROSEMARY MURPHY

From the Department of Zoology and Physiology, Wellesley College

Received for publication July 15, 1937

The present paper is a report of the production of hypertrophy of the kidney in albino rats by the metabolic stimulant, sodium dinitrophenol, and the failure to prevent the condition by the administration of large amounts of crystalline vitamin B_1. Hypertrophy of the kidney has been reported by Hoskins (1) and MacKay and MacKay (2) to occur in albino rats which have been fed thyroid preparations. MacKay, Smith and Closs (3) found that the size of the kidney following the administration of thyroid material varied directly with the increase in metabolic rate. While renal enlargement from protein-rich diets and its prevention by increasing the quantity of vitamin B complex in the diet have been reported by Hartwell (4), Reader and Drummond (5), Longwell, Hill and Lewis (6), MacKay (7), and Longwell, Johnston and Hill (8) (unconfirmed by Francis, Smith and Moise (9)), we have been unable to find in the literature any reports on the effects of the vitamin B complex on the renal hypertrophy produced by elevation of the basal metabolic rate. Consequently, it seemed of interest to determine the effect on the size of the kidney of a metabolic stimulant other than thyroid preparations and to investigate the possibility of an inhibitory action by one of the factors of the vitamin B complex.

EXPERIMENTAL PROCEDURE. Sixty-seven male and female albino rats, 35 days old (85–100 grams), of the Wellesley College stock were used for the experiment. They were kept in individual wire cages and body weights and food intake recorded daily. The rats were separated into nine groups and maintained on diets containing varied amounts of sodium dinitrophenol with and without supplementation by injections of crystalline vitamin B_1.

Pulverized Purina Dog Chow was used as the diet for the control animals and as the basis for the experimental rations. The latter consisted of the pulverized Chow to which sodium dinitrophenol (DNP) was added in the desired amounts by dissolving the DNP in 95 per cent alcohol, thoroughly mixing the solution with the Chow, evaporating the alcohol, and repulverizing the food. In this manner rations containing 0.25 per

cent, 0.3 per cent, and 0.5 per cent DNP were prepared. The control and experimental diets were distributed among the nine groups as follows: group Ia, 0.25 per cent DNP diet; group Ib, 0.25 per cent DNP diet plus daily subcutaneous injections of 150 gamma of synthetic crystalline vitamin B_1 hydrochloride (Betaxin[1]); group Ic, pulverized Chow restricted in quantity to the average amount of food consumed by the rats in groups Ia and Ib; group IIa, 0.3 per cent DNP diet; group IIb, 0.3 per cent DNP diet plus daily injections of 150 gamma of Betaxin; group IIc, pulverized Chow restricted in amount to the quantity of food consumed by the animals of groups IIa and IIb; group IIIa, 0.5 per cent DNP diet; group IIIb, 0.5 per cent DNP diet plus daily injections of 150 gamma of Betaxin; and group IIIc, pulverized Chow restricted in quantity to the food consumption of rats in groups IIIa and IIIb.

After the animals of group I and group II had been on the diets for thirty days they were killed by gas and the weights of the two kidneys and of the heart were determined as rapidly as possible. The animals in group III were killed on the fifth day since that was approximately the survival time for rats on a diet containing 0.5 per cent dinitrophenol. The surface area was calculated from the final weight on the basis of the formula, S equals $11.36 \times W^{\frac{2}{3}}$ (10). From the average daily food intake the consumption of sodium dinitrophenol was determined and expressed as milligrams per 100 sq. cm. of body surface per day.

Because of the great differences in the body weights and nutritive conditions of the rats the kidney weight-heart weight ratio was used to express variations in the size of the kidney rather than a calculation based on body weight or surface area. The ratio was found by dividing the average weight of the two kidneys by the weight of the heart.

Results. The results of the experiment are summarized in table 1. It is apparent that there was considerable variation in the response of the rats to the three diets as evidenced by differences in rate of growth, consumption of food, DNP intake, and size of kidney. The animals on the 0.25 per cent DNP diet gained weight but at a rate slightly less than one-half that of their restricted controls. The food consumption of group I was greater than that of group II but, as a result of the difference in the concentration of DNP in the diet, the dinitrophenol intake of group I was somewhat lower. On the 0.3 per cent DNP diet the rats just maintained their weight, although the food intake was sufficient to produce a 70 per cent increase in weight as evidenced by the growth of control group IIc, which was restricted to the same food consumptions as groups IIa and IIb. The rats in group III on the diet containing the highest concentration of DNP showed drastic restriction in food intake and rapid loss of

[1] A product of the Winthrop Chemical Company, Inc., New York.

weight. Because of the reduction in food this group actually consumed the lowest amount of dinitrophenol.

Examination of the values of the kidney weight-heart weight ratios indicates that the size of the kidney increased as the consumption of dinitrophenol increased. A linear relationship becomes apparent when the ratios are plotted against the DNP consumption expressed as milligrams per 100 sq. cm. of body surface per day (fig. 1). In every case the

TABLE 1

Growth rate, food intake, DNP consumption, and kidney weight-heart weight ratios on diets containing varying amounts of sodium dinitrophenol

GROUP	NUMBER OF ANIMALS	INITIAL WEIGHT	FINAL WEIGHT	INCREASE IN BODY WEIGHT	SURFACE AREA	FOOD INTAKE PER DAY	DNP INTAKE PER DAY	DNP INTAKE PER 100 SQ. CM. PER DAY	KW/HW	DIET
		grams	*grams*	*per cent*	*sq. cm.*	*grams*	*mgm.*	*mgm.*		
Ia	7	85	120	41.1	276	10.6	26.5	9.6	1.6	0.25 per cent DNP diet, no supplement
Ib	7	88	124	40.9	282	10.8	27.0	9.57	1.57	0.25 per cent DNP diet plus vitamin B_1 daily
Ic	7	86	160	86.0		10.7	0	0	1.27	Chow restricted to intake of Ia and Ib
IIa	7	94	95	1.0	237	9.5	28.5	12.0	1.7	0.3 per cent DNP diet, no supplement
IIb	8	92	101	9.7	246	9.6	28.8	11.7	1.66	0.3 per cent DNP diet plus vitamin B_1 daily
IIc	7	100	170	70.0		9.6	0	0	1.24	Chow restricted to intake of IIa and IIb
IIIa	9	89	63	−29.2	179	2.4	12.0	6.7	1.5	0.5 per cent DNP diet, no supplement
IIIb	8	92	59	−35.8	172	2.2	11.0	6.4	1.5	0.5 per cent DNP diet plus vitamin B_1 daily
IIIc	7	99	68	−31.3		2.4	0	0	1.3	Chow restricted to intake of IIIa and IIIb

ratios are greater for the animals consuming dinitrophenol than for the control animals which ingested the same quantity of food and, therefore, the same amount of protein, since, on each diet, the rats which received large doses of vitamin B_1 had approximately the same food intake, rate of growth, and kidney weight-heart weight ratios as the uninjected rats given sodium dinitrophenol.

Discussion. From these data it is apparent that upon the continuous ingestion of the metabolic stimulant, sodium dinitrophenol, there is an

increase in the kidney weight-heart weight ratio in the albino rat. Three possible explanations of the renal hypertrophy suggest themselves, an increase in the endogenous protein metabolism, a specific effect of the dinitrophenol on kidney tissue, or an increase in the maintenance metabolism. While it is not possible at the present time to state which, if any, of these explanations is correct, the similarity between these results and those of MacKay and his associates (2) (3) with desiccated thyroid indicates that variations in the basal metabolic rate may be among the factors involved. However, it may be concluded that the drug does not produce

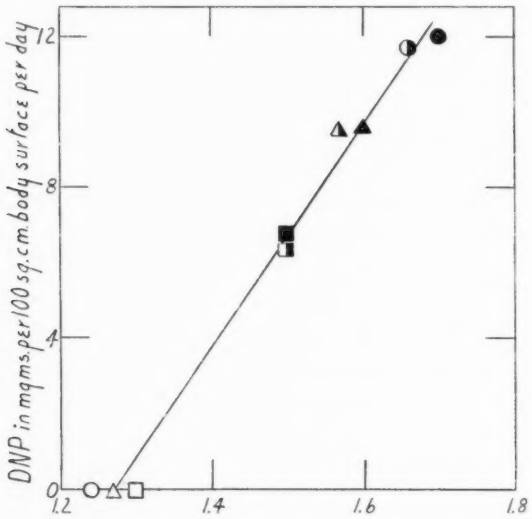


Fig. 1. Graph showing the relation of the kidney weight-heart weight ratio to the average daily consumption of sodium dinitrophenol. ▲, group Ia; ◮, group Ib; △, group Ic; ●, group IIa; ◑, group IIb; ○, group IIc; ■, group IIIa; ◨, group IIIb; □, group IIIc.

its effect through an increase in the bodily requirement for vitamin B_1 since daily injections of large doses of the crystalline vitamin did not prevent the enlargement of the kidney.

SUMMARY

The kidney weight-heart weight ratios of albino rats ingesting diets containing 0.25 per cent, 0.3 per cent and 0.5 per cent sodium dinitrophenol vary linearly with the quantity of the drug consumed. The renal hypertrophy is not prevented by large doses of synthetic crystalline vitamin B_1.

REFERENCES

(1) Hoskins, E. R. J. Exper. Zool. **21:** 295, 1916.
(2) MacKay, E. M. and L. L. MacKay. J. Nutrition **4:** 33, 1931.
(3) MacKay, E. M., F. M. Smith and K. Closs. J. Nutrition **6:** 151, 1933.
(4) Hartwell, G. A. Biochem. J. **22:** 1212, 1928.
(5) Reader, V. and J. C. Drummond. Biochem. J. **20:** 1256, 1926.
(6) Longwell, B. B., R. M. Hill and R. C. Lewis. J. Nutrition **5:** 539, 1932.
(7) MacKay, E. M. This Journal **106:** 571, 1933.
(8) Longwell, B. B., R. P. Johnston and R. M. Hill. J. Nutrition **12:** 155, 1936.
(9) Francis, L. D., A. H. Smith and T. S. Moise. This Journal **97:** 210, 1931.
(10) Mitchell, H. H. and G. G. Carman. This Journal **76:** 385, 1926.

THE EFFECT OF WATER DEPRIVATION ON THE FLUID EXCHANGE OF CATS WITH DIABETES INSIPIDUS[1]

C. FISHER, H. W. MAGOUN AND A. HETHERINGTON

From the Institute of Neurology, Northwestern University Medical School

Received for publication July 15, 1937

In a previous paper the effect of restricting the water intake of cats with experimental diabetes insipidus to the pre-operative level for several days during the permanent phase has been reported (Fisher, Ingram and Ranson, 1935). At this time observations made on a much larger series of polyuric animals and under a variety of experimental conditions will be presented. Of particular interest are the results obtained from animals which were deprived of water for a period of weeks. So far as we know, the effect of long-continued water restriction on the fluid exchange of diabetes insipidus has never been investigated. A series of twenty cats was used in these studies. Experimental diabetes insipidus was produced in these animals with the aid of the Horsley-Clarke stereotaxic instrument in the same manner as previously described (Fisher, Ingram and Ranson, 1935).

On the basis of an analysis of 85 cases of experimental diabetes insipidus in the cat it has been found that the disturbance in the fluid exchange develops in the great majority of cases in two phases (Fisher, Ingram and Ranson, 1938). A transient polyuria and polydipsia usually makes its appearance on the first post-operative day and lasts for four or five days. This is followed by a period of normal water balance. Then, around the fourteenth post-operative day there is another increase in the fluid intake and urine output which persists indefinitely. The temporary increase during the first few post-operative days has been called the *transient phase* of the diabetes insipidus and the persistent increase which appears in about two weeks the *permanent phase*. The period of normal water balance between these two phases has been designated the *normal interphase*.

The various experimental procedures used will be best described in the presentation of results.

The effect of water deprivation from the time of operation to the onset of the permanent phase. A series of 14 operated cats were deprived of all extra-

[1] Aided by a grant from the Rockefeller Foundation.

dietary water from the day of operation to the onset of the permanent phase. This was the equivalent of restricting the cats to their pre-operative fluid intake, for normal caged cats fed on a diet consisting of 60 grams of beef hearts and 100 cc. of milk do not drink water. Seven of these developed diabetes insipidus, representing somewhat better than average success. The purpose of this experiment was to see if the transient and permanent phases of the polyuria would develop independently of the water intake. The cats were offered water each morning but only permitted to drink a few cubic centimeters in order to ascertain whether there was any correlation between the development of thirst and the appearance of the two phases of the polyuria.

The effect of water deprivation in these cats during the transient phase was not very clear-cut. When offered water for a few minutes all but one of the seven cats showed thirst for a few days in the early post-operative period corresponding to the time when the transient phase ordinarily occurs. Although in a number of cases the amount of urine excreted was somewhat excessive (180–185 cc.) and the specific gravity of the urine rather low (1.020–1.023) during the period when the cats were manifesting thirst, there was no marked deviation from the normal. And, since cats with hypothalamic lesions which do not develop the transient phase of diabetes insipidus occasionally show urine outputs on the first post-operative day as great as that shown by any of these cats, we do not feel justified in concluding from these data that the transient phase of the polyuria can occur in the absence of free water.

It was found that at the end of a latent period varying from ten to fourteen days the urine outputs of the seven cats showed a precipitous increase and the specific gravity showed a marked fall even though the animals were prevented from drinking water. In three instances the urine output rose above 300 cc., a negative water balance of more than 200 cc. thus developing in each case. Not until these animals manifested thirst when given water were they given a free supply of water. In two instances thirst was displayed on the second day of the permanent phase, in four cats on the third day and in one on the fifth day. One animal (cat 1) continued to excrete excessive amounts of urine for the first four days of the permanent phase even though no water was allowed, a negative water balance of 410 cc. developing during this period. We do not wish to imply that these seven animals would have waited two to five days before beginning to compensate for their polyurias if permitted free water, for in our experience it has been found that ordinarily compensation takes place much sooner. However, when offered water for a few minutes once a day these cats showed thirst for the first time on the days indicated. The average fluid exchanges of these animals during the ten to fourteen days preceding the onset of the permanent phase were well within the normal limits.

The effect of restricting the water intake to the pre-operative level for several days during the permanent phase. Three animals (cats 2, 3 and 4) whose average daily fluid exchanges ran over 400 cc. were restricted to their pre-operative fluid intake, 100 cc. of milk and no water, for several days during the permanent phase. When this was done the output of urine in the cases of cats 2 and 3 decreased rapidly and approached normal values after two or three days, while the specific gravity of the urine rose considerably. But, although the urine output tended to approach normal, a considerable negative water balance developed in each case. Thus, cat 2 excreted 90 cc. more urine than the fluid she took (in the form of milk) during the three days of water deprivation, while the negative water balance amounted to 120 cc. over a period of two days in the case of cat 3. It should be noted that ordinarily the fluid intake in the form of water or milk is somewhat in excess of the urine output. When free water was again permitted the polyuria and polydipsia returned immediately and in both instances (2, 3) a positive water balance developed on the first day which was approximately equivalent to the negative water balance developed during the period of restriction. In other words, the water loss was immediately made up as soon as the cats were permitted free water.

Cat 4 is of especial interest because the results in this case show that dehydration consequent upon water deprivation can be carried to such a point that the animal will die. In this instance we were dealing with an animal with a very marked diabetes insipidus, sometimes the daily fluid exchange amounting to very near a liter. (The fluid exchange of normal cats on the same diet amounts to about 100 cc. daily.) The animal seemed in good condition at the end of the first day of water deprivation even though there had developed a negative water balance of 325 cc., the urine output on this day being 425 cc. At the end of the second day of thirst the cat was found in a comatose state and could not be revived. At this time the total negative water balance amounted to 450 cc., 225 cc. more urine having been excreted.

The effect of gradual restriction of the water intake for a period of weeks during the permanent phase. Two cats (5 and 6) were subjected to periods of gradual water restriction. Before the experiment both these cats were running marked permanent phases, in each instance the average daily fluid exchange amounting to more than 400 cc. Cat 5 was restricted to about 300 cc. of fluid (100 cc. of milk and the balance water) for eight days, to 200 cc. for four days; given free water for two days and then deprived of all extra-dietary water for two days. Cat 6 was restricted to about 300 cc. for eight days, to 200 cc. for nine days; given free water for four days and then deprived of all water (except for that present in the meat and milk) for seven days. It was found that with gradual water restriction there was a corresponding fall in urine output but that the output and

intake closely approximated one another whereas the fluid intake is generally somewhat in excess of the urine output when the animals are given free water. Thus during the nine day period when the fluid intake of cat 6 was restricted to 200 cc. the difference between the average intake and output amounted to only 9 cc. (200 cc. against 191 cc.), while before the experiment the intake was 77 cc. in excess of the output. On the first day of free water both the cats developed a positive water balance of about 140 cc. suggesting that during the period of restriction there was excessive loss of water through the kidney. The behavior of the fluid exchange in these two cats when the water intake was restricted to the pre-operative level was the same as for the animals described in the first section. However, cat 6 was deprived of all its extra-dietary water for seven days. On the seventh day the animal manifested signs of illness so that it was thought advisable to give it water. During this week the fluid exchange was within normal limits but a negative water balance developed at the beginning of the week and a positive balance when free water was given at the end.

The effect of prolonged water deprivation during the permanent phase. The two cats (5 and 6) which were subjected to gradual water restriction at an earlier period and six additional animals were totally deprived of extra-dietary water for a period of twenty-one days. Those that survived this experiment were then deprived of both water and milk for an additional eight days but given the usual amount of meat. Before starting the experiment the diet of these cats was regulated to an amount only slightly in excess of that required to maintain a constant weight. Five of the animals utilized in these experiments had fluid exchanges varying from 400 to 600 cc. daily (5, 6, 7, 8 and 9); two of them had fluid exchanges varying from 300 to 400 cc. daily (10 and 11); and one had a mild diabetes insipidus, the fluid exchange ranging from 100 to 200 cc. daily (12). During the period of water deprivation the animals were carefully weighed each morning.

Seven of the eight cats survived the twenty-one day period of extra-dietary water deprivation. The behavior of the fluid exchange and its relation to changes in body weight during the prolonged period of water restriction were very interesting. On the first day of water deprivation the five animals with the most marked diabetes insipidus (cats 7, 6, 8, 9 and 5) showed a decrease in the urine output but, as in the thirst experiments of short duration described above, not a return to the normal level. In each instance a considerable negative water balance developed, varying from 75 cc. in the case of 5 to 195 cc. in the case of 8. Most important, each cat showed a loss in weight which was proportional to the water loss sustained. Thus, cat 7 had a negative water balance of 165 cc. and a weight loss of 175 grams, cat 9 had a negative water balance of 165 cc. and

lost 160 grams, and the corresponding figures for 8 were 195 cc. and 205 grams respectively. In the remaining three cases in which the diabetes insipidus was less marked, only slight negative water balances developed and the weight losses were correspondingly low. Thus, cat 10 lost 35 cc. of water and 65 grams of weight, 12 lost 25 cc. of water and 30 grams of weight and 11 showed no negative water balance and lost 15 grams of weight.

One of the cats with the more marked degrees of diabetes insipidus (cat 6) died on the eleventh day of water deprivation. Several days before its death the animal stopped eating its meat and became quite weak. On the morning of the eleventh day it was found dead in its cage. It would appear that this animal died of dehydration.

Excluding the first day of the experiment on which the negative water balance developed, these cats showed a quite normal water balance during the remaining twenty days of extra-dietary water deprivation. It is evident, therefore, that cats with diabetes insipidus can be deprived for long periods of the excessive amounts of water which they ordinarily drink without showing any ill effects. In the seven cats which survived the experiment there was no indication of a tendency toward increased urine excretion during these twenty days. Further, the behavior of the weights of these animals during this period did not reveal the presence of such a tendency. Five of the animals which ate all their food showed a slow increase in weight and at the end of the twenty-one day period had regained enough weight to compensate for the initial drop, when such occurred, on the first day of water restriction. These increases were due to the fact that the diet given was somewhat more than sufficient to maintain the weights of the cats.

These seven surviving cats were next deprived of their milk as well as their water for an additional eight days, receiving only meat. Five of them survived this treatment, including two (7 and 9) with marked diabetes insipidus. The average urine output of these animals was decreased by half or less over what it was on the full diet and the specific gravities showed corresponding increases. Those cats which originally had the most marked degrees of diabetes insipidus showed some tendency toward excessive water loss during this period. Three normal cats were deprived of milk and water for eight days but given the same amount of meat as the diabetic cats. The urine outputs for these animals were 36(1.063), 29(1.066) and 38(1.061) respectively. On the other hand, cats 7 and 9 had average urine outputs of 59(1.045) and 48(1.044) cc. respectively during the eight day period. The three remaining cats (10, 12 and 11), which had less marked diabetes insipidus and which survived the total fluid restriction, showed urine outputs comparable to those for the normal animals, although the specific gravities were somewhat lower. Although

the data were not highly consistent, there was some indication that the polyuric animals which had the highest urine outputs during this period lost the most weight, and further, lost more weight than did the normal cats.

Two of the cats (5 and 8) became moribund after a few days of combined water and milk deprivation. Although it ate no food whatsoever on the first and second days of the experiment, cat 8 excreted 45 cc. of urine each day. On the third day it was so weak that it was thought advisable to kill it. Cat 5 likewise developed anorexia but on the first, second and third days excreted 50, 45 and 45 cc. of urine respectively. On the fourth day it was killed when it appeared that it would not live through the night.

At the end of the eight day period the five surviving cats were given free water. In each case the fluid exchange showed a marked increase, exceeding in every instance the level obtaining during the period preceding the experiments. More important the fluid intake in the cases of cats 7 and 9 was considerably in excess of the urine output, the positive water balance amounting to 185 cc. for the former and 115 cc. for the latter. At the same time both cats showed weight increases of corresponding magnitudes, 210 grams in the case of 7 and 80 grams in the case of 9. For both cats, especially 7, the positive water balance and the gain in weight tended to approximate the negative water balance and the loss of weight registered on the first day of extra-dietary water deprivation twenty-nine days before. Further, these positive findings are confirmed by the negative ones from the three remaining cats (10, 12 and 11). In these cases the positive water balances and the gains in weight were hardly significant and likewise the negative water balances and the weight losses twenty-nine days before were quite small. Thus, cat 11 had a positive water balance of 20 cc. and gained 30 grams in weight. It would appear, therefore, that cats 7 and 9 were in a chronic state of water deficit all through the twenty-nine days of water deprivation, but when given free water immediately made up the loss. When left on free water all five cats tended to return to the level of fluid exchange prevailing before the start of the experiment.

The effect of giving water by stomach tube on the polydipsia of the permanent phase. Two cats (13 and 14) which had marked diabetes insipidus (daily fluid exchange over 600 cc.) were given the amount of water they ordinarily drank over a sixteen hour period by stomach tube. The water was administered in doses of 100 cc. at intervals of about four hours, but the cats were permitted free water. The purpose of this experiment was to see if it was necessary for the excessive amounts of water ingested by these animals in the ordinary course of the permanent phase to pass through and moisten the mucosa of the buccal and pharyngeal cavities in order to satisfy the thirst of the animal. If water by stomach tube prevented the animal from drinking, it would indicate that the thirst of

diabetes insipidus can be satisfied independently of the moistening of the buccal and pharyngeal membranes. On the other hand, if the cat continued to drink its usual amount of water it would suggest that a pathological thirst existed entirely independent of bodily hydration.

The results showed clearly that, whereas cat 13 ordinarily drank 350 to 450 cc. of water during the sixteen hour period between 8 a.m. and midnight, when given an equivalent amount of water by stomach tube it drank only 75 cc. Further, during the first nine hours no water was drunk. Between 5 p.m. and 10 p.m. the 75 cc. of water were ingested. This was because the cat was fed at 5 p.m. and we have observed that polyuric animals drink a great deal in the period following feeding. By spacing the stomach tubings so as to provide more water in the period immediately following feeding, we were able to cut down the water intake of cat 14 to 30 cc. This small amount was drunk in the last two and a half hours of the experiment. It was shown that simply passing the stomach tube a number of times during a sixteen hour period had no effect on the polydipsia.

Discussion. A number of conclusions can be drawn from these experiments on water deprivation. First, and most important, they indicate that the polyuria of the permanent phase of diabetes insipidus is primary to the polydipsia. The first series of experiments needs little comment. They showed clearly that the onset of the permanent phase of the polyuria can develop when the fluid intake is restricted to the pre-operative level of normal cats, e.g., 100 cc. of milk.

No definite conclusions as to the effect of water deprivation on the development of the transient phase are forthcoming. Our results do not warrant the conclusion that it can develop in the absence of free water; neither can one say that under these conditions the transient phase will not develop. For, from the very nature of the experiment, it is not known whether the transient phase would have developed even if free water were provided, and if it did develop how severe it would have been. However, it must be said that it is probable that a well marked transient phase would have made its appearance in some of the cats if free water were given because a transient phase of varying degrees has occurred in at least 75 per cent of our large series of cases. Several factors may have prevented the appearance of the transient phase. On the day of operation the cats received no food but were injected with 100 cc. of normal salt solution. Further, they were subjected to a brain operation and a general anesthetic. These factors may have combined to prevent the appearance of the transient phase when the cats were deprived of water. During the permanent phase, it will be recalled, the cats showed an excessive urine output when deprived of water for only a day or at most two days, even though they were in good health, well-nourished and well hydrated at the time the

thirst experiment was begun. It is clear that further work needs to be done on the transient phase. It may be pointed out that Richter (1935) and Richter and Eckert (1935) were able to elicit the polyuria of the transient phase in the rat immediately following operation, even though water was withheld.

In the second series of experiments it was shown that, although the fluid exchange of diabetic cats falls to normal after several days of water deprivation, a negative water balance develops which is immediately compensated for by a positive water balance as soon as free water is offered again. The third group of experiments revealed that gradual water restriction brings about a decrease in the urine output proportionate to the decrease in the fluid intake, and that animals can tolerate such restriction for long periods. Further, the fluid intake in these cases approximated the urine output much more closely than during the ordinary course of the diabetes insipidus, when the former usually exceeded the latter by a good deal. This approximation was probably due in large part to the tendency toward excessive and continuous water loss through the kidney displayed by polyuric animals. That such a loss occurred was evidenced by the building up of a positive water balance by the cats the first day they received free water. Possibly this excessive renal water loss takes place at the expense of extra-renal elimination.

Another group of experiments showed that cats with marked diabetes insipidus can be totally deprived of the excessive amounts of water which they ordinarily drink and survive for periods as long as three weeks. Further, some of the animals were able to tolerate an additional eight days without any fluid aside from the water content of the meat. Again on the first day of water deprivation the cats with the more marked degrees of diabetes insipidus developed negative water balances, and corresponding losses in body weight indicating the loss of body water. The evidence showed that these animals were in a chronic state of water deficit which was made up when free water was given, a gain of weight being registered which corresponded to the development of a positive water balance.

The question arises, why did not all the animals become progressively dehydrated if diabetes insipidus actually represents a condition in which there is a tendency toward renal water loss. At the present time a final answer to this question cannot be given. However, it will be remembered that four of the cats did die or became moribund. The most convincing case is that of cat 4. This animal had a more marked degree of diabetes insipidus than did any of the animals used. The degree of the negative water balance which develops after the first day or two of water deprivation seems to depend upon the severity of the diabetes insipidus present beforehand. Thus, cat 4 lost 450 cc. of body water in two days. It should be emphasized that this is not a negligible loss but represents about 15

per cent of the body weight. Apparently this is a dangerous degree of dehydration for a cat. None of the other cats lost more than about 200 cc. of water, but even this is 6 to 7 per cent of the body weight. The three other cats (6, 5 and 8) which died or became moribund belonged to the group of animals with the more severe degrees of diabetes insipidus and were also cats which had developed considerable negative water balances. Cat 6 died after eleven days of water deprivation and showed symptoms of dehydration. The other two cats became very ill after several days of combined water and milk deprivation and were killed. The cause of illness in these animals is not clear. All of these three cats stopped eating their meat during the period of illness. This anorexia we believe, was the result of the illness and not its cause, for we have shown that normal cats can be deprived of their milk and water for a period of eight days, receiving only meat, without showing any bad effects. Further, cats 7 and 9 which developed marked negative water balances, also were able to survive the full twenty-nine days of water deprivation.

It appears, therefore, that if the loss of body water on the first day or two following water deprivation is not too great, the animal has a good chance of surviving for many days. If, on the other hand, there is a marked loss during the first few days, as in the case of cat 4, dehydration may lead to a fatal outcome. And even in those animals where the initial water loss is not excessive, as in cats 6, 5 and 8, death may occur after a variable number of days.

During the twenty-one days of water deprivation the seven cats showed fluid exchanges which were quite within the normal range. It would seem, therefore, that there are mechanisms other than the supraoptico-hypophyseal system which can prevent undue water loss in animals with diabetes insipidus. Or the supraoptico-hypophyseal system may be partially intact and able to compensate. Thus, cats 10 and 11 which had somewhat less severe degrees of diabetes insipidus than did those animals which died, and presumably less destruction of the supraoptico-hypophyseal system, survived the full period of water deprivation and showed little evidence of water loss at the beginning of the experiment.

The fact that the urine output in diabetic cats may return to a normal level during water deprivation is no proof that the polydipsia is primary, as many have held. The antidiuretic hormone of the neural lobe of the hypophysis is not the only factor preventing loss of water through the kidneys. The evidence indicates that this hormone acts on the tubules to increase water reabsorption. It is not, however, responsible for the full degree of water reabsorption in the tubules. Thus, according to Cushny (1926) it has been calculated that in the cat about 12,000 cc. of fluid filter into the tubules in twenty-four hours out of which 11,900 cc. are reabsorbed and 100 cc. excreted as urine. In the absence of the antidiuretic

hormone, as in diabetes insipidus, rarely more than one liter of urine is excreted, which means that this principle accounts for the reabsorption of not more than 1,000 cc. of water in the tubules. It is evident that reabsorption can go on independently of the antidiuretic hormone, and that this hormone is only one of the factors responsible for reabsorption. It is clear that further work has to be done to ascertain just what happens in the diabetic animal during water deprivation, e.g., blood chemical studies.

Those who consider that diabetes insipidus is essentially a pathological polydipsia have never made it clear just what is meant by this designation. One interpretation is that there is a heightened sensation of thirst which is independent of bodily hydration and that the diabetic animal has a polyuria simply because it drinks excessively. If this pathological thirst is localized in the buccal and pharyngeal mucosa simply wetting the mouth should satisfy it and conversely water which enters the body but does not moisten these membranes should have no effect on the polydipsia. The experiments on cats 13 and 14, described above, definitely show that the thirst of diabetes insipidus can be relieved by giving the animal water by stomach tube. This does not conflict with Cannon's theory of thirst, for Cannon holds the thirst is a dryness of the mouth due to failure of secretion of the salivary glands when the body is dehydrated.

The continuation of the polyuria of diabetes insipidus for a day or two in spite of water deprivation confirms the results of Camus and Roussy (1914), Bailey and Bremer (1921), Bourquin (1926) and Richter (1935) and Richter and Eckert (1935). Water deprivation for many days, as in our experiments, has rarely been attempted. Curtis (1924) restricted the fluid intake of his dog F15 for a week without ill effects. In this animal there was some indication of water loss in excess of that before operation. The figures show that on the first day of thirst, when the water was cut down to 400 cc., the urine output was 350(1.015) cc., while before operation the average fluid exchange for a four day period was 225(1.050)–420 cc. In man it is, of course, very difficult to restrict the water intake for long periods because of the discomfort suffered by the patient. However, when water deprivation is carried out for a number of hours, the continuance of the polyuria can be observed. Thus, Karlson and Norberg (1936) report that in one of their patients with an eight to thirteen liter diabetes insipidus, three liters were excreted during an eight hour period of water deprivation.

SUMMARY

Evidence is presented which indicates that the polyuria of diabetes insipidus is primary to the polydipsia. The permanent phase of the polyuria makes its appearance after the usual latent period although the fluid intake is restricted to the pre-operative level. Under these conditions or

when the fluid intake is similarly restricted after the permanent phase has become established, a negative water balance is developed. When later water is supplied freely this negative balance is compensated for by the development of a positive water balance.

During the permanent phase the fluid intake of polyuric cats may be restricted to the pre-operative level for periods of weeks without apparent harm to the animal. Such restriction is associated with the development of a negative water balance at the beginning of the experiment, but aside from the first day the fluid exchange remains within normal limits. When free water is given again a positive water balance develops, indicating that during the period of deprivation the cats were in a state of chronic water deficit. In some cases where the polyuria is unusually severe or where the restriction of fluid is below the pre-operative level, the diabetic cats are unable to survive, although normal cats suffer no ill effects from the same degree of fluid restriction.

REFERENCES

BAILEY, P. AND F. BREMER. Arch. Int. Med. **28:** 773, 1921.

BOURQUIN, H. This Journal **79:** 362, 1926.

CAMUS, J. ET G. ROUSSY. Compt. rend Soc. de biol. **76:** 121, 1914.

CURTIS, G. M. Arch. Int. Med. **34:** 801, 1924.

CUSHNY, A. R. The secretion of urine. Longmans, Green and Co., Ltd., London, 1926.

FISHER, C., W. R. INGRAM AND S. W. RANSON. Arch. Neurol. and Psychiat. **34:** 124, 1935.

FISHER, C. Diabetes insipidus and the neuro-hormonal control of water balance: A contribution to the structure and function of the hypothalamico-hypophyseal system. Oxford University Press, 1938.

KARLSON, S. AND B. NORBERG. Acta Med. Scand. **88:** 585, 1936.

RICHTER, C. P. This Journal **112:** 481, 1935.

RICHTER, C. P. AND J. F. ECKERT. This Journal **113:** 578, 1935.

MUSCULAR EFFICIENCY IN RELATION TO THE TAKING OF FOOD AND TO THE HEIGHT OF THE RESPIRATORY QUOTIENT IMMEDIATELY BEFORE EXERCISE[1]

JOHN HALDI, GEORGE BACHMANN, CHARLES ENSOR AND WINFREY WYNN

From the T. T. Fishburne Laboratory of Physiology, Emory University, Emory University, Georgia

Received for publication July 19, 1937

In a recent monograph Haggard and Greenberg (1935) reported an increase in muscular efficiency after meals; thus, in one experiment it rose from 19.5 per cent in the fasting state to 27.0 per cent one hour after breakfast. This increase in efficiency, they believe, was related to a rise in the respiratory quotient and to an increase in the utilization of carbohydrate at rest immediately before exercise. They therefore concluded that fluctuations of the respiratory quotient are accompanied by similar fluctuations in muscular efficiency.

In a study of the respiratory exchange following the ingestion of glucose and of fructose in which we have been engaged for some time, we found in agreement with Carpenter and Fox (1931a) that there was no increase in muscular efficiency when exercise was taken immediately after the ingestion of 50 grams of glucose or of fructose, or a mixture of 25 grams each of these sugars. These observations were made on two subjects in a total of 24 experiments. When work was done for two consecutive 15 minute periods at the rate of 550 kgm.-m. per minute, the muscular efficiency after the ingestion of the sugars was the same as in the control experiments in which water alone was taken. While these experiments led us to believe that, normally, muscular efficiency is not increased by the taking of food, the objection may be raised that there was no time allowed for absorption before beginning the exercise. It was therefore decided to carry the investigation further under conditions more nearly comparable to those of the experiments of Haggard and Greenberg.

METHOD. The subjects of these experiments were two male adults. Both were well trained for this kind of work, having served as subjects in a large number of experiments of this nature. Three series of experiments were conducted in which exercise was taken 1, 30 minutes after the ingestion of water (controls), glucose, fructose, or a mixture of these

[1] Preliminary Report, This Journal Proc. **119:** 323, 1937.

sugars in equal parts; 2, in the fasting state and again one, two, and three hours after the ingestion of a mixture of glucose and fructose; 3, before, and one, two, and three hours after breakfast.

For the sake of clarity those details of the procedure which were different in the various types of experiments will be described immediately before the presentation of the results. The respiratory exchange was determined in all the experiments by the open circuit method of Carpenter and Fox (1931b). Aliquot samples of the expired air were collected in large rubber bags, the sample passing from the main stream into the bag through an opening 1.04 mm. in diameter (*cf.* Bachmann and Haldi, 1937a). The analyses were made by two analysts, and were required to check within 0.02 per cent. Exercise was taken on a Prony brake bicycle ergometer either for 15 or 30 minutes, work being done at a steady rate of 550 kgm.-m. per minute. The tension of the spring on the wheel of the ergometer was maintained constant and pedalling of the bicycle was timed to the beat of a metronome. In those experiments in which the respiratory exchange was determined during recovery, the subject slid off the bicycle at the end of exercise on to an adjacent couch without removing the mouthpiece; the experiment was, therefore, continued uninterrupted. The non-protein respiratory quotient used in the determination of the heat production was calculated on the basis of the average hourly nitrogen excretion of each subject obtained from a large number of experiments. The subject came to the laboratory in the fasting state at about 7 a.m., the last meal having been taken not later than 7 o'clock the evening before. He then reclined for 30 minutes before beginning the experiment.

Results. *Series 1.* This series included 24 experiments. After the usual preliminary rest, the basal respiratory exchange was determined for 45 minutes with the subject in the recumbent position. Twenty grams of glucose, fructose, or a 20 gram mixture of these sugars in equal parts dissolved in 200 cc. water at 37°C. were then ingested in the sitting posture. Control experiments were done with water alone. After ingestion, the prone position was resumed and the respiratory exchange determined for 30 minutes. The subject then arose, mounted the bicycle, inserted the mouthpiece and at a given signal began the work which was continued for 30 minutes. Upon completion of the exercise the determination of the respiratory exchange was continued with the subject lying at rest for one hour and 15 minutes to allow for complete recovery. The samples of expired air were collected and the meter read at intervals of 15 minutes.

The results are shown in table 1. For reasons to be given later, the muscular efficiency as presented in the table, has been calculated first by omitting, and secondly, by including the excess oxygen of recovery. The

net efficiency was determined from the difference between the oxygen utilized during exercise (or exercise and recovery) and that consumed while lying at rest for one half-hour before exercise. The basal respiratory quotients which are not included in the table were fairly regular, ranging from 0.76 to 0.80. In the glucose experiments the respiratory quotient immediately before exercise was practically at the basal level, whereas in those with fructose and a mixture of the sugars it was appreciably higher, reaching 0.90 in one group of experiments. As shown in the table, there was, however, no significant difference between the muscular efficiency after the ingestion of the sugars and that of the control experiments

TABLE 1

*Respiratory quotients before exercise and net muscular efficiency when exercise (of one-half hour duration) was begun 30 minutes after ingestion**

SUBSTANCE INGESTED	R.Q. BEFORE EXERCISE†	NET MUSCULAR EFFICIENCY	
		Excess O_2 of recovery omitted	Excess O_2 of recovery included
Subject W. W.			
		per cent	*per cent*
200 cc. water (control experiments)	0.79	23.1	21.9
20 grams glucose	0.79	22.8	21.7
20 grams fructose	0.86	23.2	22.1
10 grams glucose; 10 grams fructose	0.89	24.0	22.9
Subject C. E.			
200 cc. water (control experiments)	0.77	21.6	20.3
20 grams glucose	0.76	21.1	20.1
20 grams fructose	0.86	21.5	20.6
10 grams glucose; 10 grams fructose	0.90	21.9	20.8

* Each value in the table is an average of three experiments.

† In recumbent position.

with water and, consequently, no correlation between muscular efficiency and the respiratory quotient of the resting state before exercise. These results are at variance with those of Haggard and Greenberg who found a marked increase in muscular efficiency when the resting respiratory quotient rose above 0.85. In this connection it may be noted that more carbohydrate was oxidized during exercise after ingestion of the sugars than in the control experiments (Bachmann and Haldi, 1937b).

Series 2. While the above experiments show the same muscular efficiency after the ingestion of the sugars as in the controls, they offer no comparison between the efficiency before and after the ingestion of the sugars. In the present series the efficiency was determined in the fasting

state and one, two, and three hours after taking a mixture of 25 grams glucose and 25 grams fructose. Controls were run in which neither food nor water was taken. Two experiments with the sugars and three controls were done on each subject, making a total of 10 experiments. The results with the sugars were so uniform that two experiments were considered sufficient for reliable averages. The respiratory exchange was determined for 10 minutes immediately before each exercise period while the subject sat at rest on the bicycle. Two minutes were allowed for becoming adjusted to the noseclip and mouthpiece before taking the initial reading on the gas meter and beginning the collection of the gas sample. After the first exercise period the sugar dissolved in 500 cc. water at 37°C. was ingested. In the interval between the ingestion of the sugar and riding the bicycle the subject carried on the routine work of the laboratory. The net muscular efficiency was determined from the difference between the minute oxygen consumption during exercise and that at rest on the bicycle.

Because of the length and strenuous conditions of the experiments it was deemed inadvisable to determine the respiratory exchange during recovery. To do so would have necessitated an almost continuous breathing into the apparatus for approximately four hours, which in all probability would have introduced the factor of fatigue. The energy expended in work was therefore determined from the oxygen consumed during exercise alone. This procedure was believed justified, inasmuch as in the experiments of table 1, the relative efficiencies were the same whether the excess oxygen of recovery was included or omitted. This may be accounted for by the extremely small variation in the excess oxygen of recovery relative to the amount of oxygen consumed during exercise.

The results are presented as averages in table 2. The respiratory quotients of the two subjects at rest on the bicycle were 0.77 and 0.76, respectively, and the net muscular efficiency during exercise 25.1 and 23.7 per cent, respectively. Approximately one hour after the ingestion of the sugars, the respiratory quotient of W. W. at rest was 0.92 and that of C. E. 0.88, while the muscular efficiency was 24.9 and 24.0 per cent, respectively. One hour later with a resting respiratory quotient of 0.81, and two hours later with quotients of 0.74 and 0.77, the muscular efficiency had not changed. Although there were considerable variations in the respiratory quotients at rest immediately before the various exercise periods the muscular efficiency showed no significant change; furthermore, it was the same at the various intervals throughout the morning after ingestion of the sugars as at the corresponding time in the control experiments with water. It is worthy of note that in the control experiments there was no decrease in muscular efficiency from 7:30 a.m. to 11:00 a.m.

Series 3. These experiments were conducted in precisely the same

manner as those of series 2 except that breakfast was taken instead of the sugar. Breakfast consisted of two slices of toast, a small amount of butter (about 7.5 grams), one egg and two slices of bacon. One subject (W. W.) took in addition a pint of milk and the other (C. E.) a cup of coffee containing 25 grams sucrose. The energy value of the meal was approximately 500 Calories with a relatively low carbohydrate content.

TABLE 2

*Net muscular efficiency before and after the ingestion of a 50 gram mixture of glucose and fructose in equal parts and before and after breakfast**

TIME AT WHICH THE 15 MINUTE EXERCISE PERIOD WAS BEGUN	SUBJECT W. W.		SUBJECT C. E.	
	R.Q. 10 minute period before exercise†	Net muscular efficiency	R.Q. 10 minute period before exercise†	Net muscular efficiency
a.m.		*per cent*		*per cent*
	No food taken—control experiments			
7:30	0.78	24.6	0.78	23.9
8:45	0.78	25.2	0.78	23.8
9:45	0.80	24.6	0.75	23.9
10:45	0.79	24.0	0.75	23.7
7:30 (fasting)	0.77	25.1	0.76	23.7
7:35		50 grams sugar ingested		
8:35	0.92	24.9		
8:50			0.88	24.0
9:35	0.81	24.9		
9:50			0.81	23.7
10:35	0.74	25.3		
10:50			0.77	23.4
7:30 (fasting)	0.76	24.6	0.78	24.4
7:50		Breakfast		
9:00	0.75	24.8	0.83	23.4
10:00	0.76	24.6	0.78	24.2
11:00	0.75	24.4	0.78	23.8

* Each value in the control and breakfast experiments is an average of three experiments; in the sugar experiments, an average of two experiments.

† Seated on bicycle.

Six experiments were done, three on each subject, the averages of which are given in table 2. The muscular efficiency was practically the the same, one, two, and three hours after as before breakfast, except that with C. E. it was one per cent lower one hour after breakfast. A difference of this magnitude, however, we believe to be of no significance. The ingestion of food of itself therefore did not lead to an increase in muscular efficiency. The table shows that the respiratory quotient does

not necessarily increase after an ordinary meal. The breakfast taken by our subjects would doubtless be regarded by many as a standard one and yet the respiratory quotient with a single exception was practically at the basal level, one, two, and three hours after the meal. When the low quotients were first observed in the early experiments the apparatus and procedure were carefully checked but no possibility of a technical error could be detected. Subsequent experiments with this type of breakfast yielded the same results. As we have obtained a considerable rise in the respiratory quotients of both subjects after a meal containing a large percentage of carbohydrate, the low quotients were doubtless due to the relatively large amount of fat and protein in the breakfast.

Six other experiments (not included in the table) were done, three on each subject, in which there were only two 15 minute exercise periods, one before and the other one hour after ingestion of *a*, carbohydrate alone (25 grams glucose and 25 grams fructose); *b*, the same breakfast as in series 3; *c*, breakfast containing more carbohydrate and less protein and fat. The high carbohydrate breakfast consisted of 200 grams bananas, 30 grams sucrose and 4 slices of toast. In addition, subject C. E. took about 200 cc. of coffee, and subject W. W. a pint of milk and 8 grams of butter. The breakfast yielded approximately 500 Calories from carbohydrate alone. The purpose of the experiments was twofold: first to compare the results obtained after taking a small and a large amount of carbohydrate; secondly, to establish definitely whether in this type of experiment as in those of table 1, the relative efficiencies would be the same when the excess oxygen of recovery was included as when omitted in the calculations. The respiratory exchange was determined in these experiments for three consecutive 10 minute periods after exercise. The respiratory quotients before and one hour after taking the sugars and the low carbohydrate breakfast were practically the same as those in the experiments of table 2; after the high carbohydrate breakfast the quotients of the two subjects rose from 0.77 to 0.84 and 0.92 respectively. These observations confirm the conclusion that the low quotients after breakfast in table 2 should be attributed to the small amount of carbohydrate in the meal. The muscular efficiency in all these experiments as in those of table 2 was substantially the same one hour after taking food as in the fasting state. When the excess oxygen was included in the calculations the relative muscular efficiencies, as in table 1, were the same as when it was omitted. The absolute muscular efficiencies, however, were approximately one per cent lower.

CONCLUSIONS

When 20 grams of glucose or fructose or a 20 gram mixture of these sugars in equal parts was ingested 30 minutes before exercise, the muscular

efficiency was the same as in the control experiments in which water alone was taken. The respiratory quotient immediately before exercise was at the basal level in the control and glucose experiments whereas it had risen approximately 0.09 and 0.13 respectively, in the experiments with fructose and a mixture of the sugars.

The muscular efficiency was the same as before ingestion one, two, and three hours after *a*, the ingestion of a 50 gram mixture of glucose and fructose in equal parts, and *b*, taking a low carbohydrate breakfast. It also remained unchanged over the same length of time in control experiments in which no food was taken. The respiratory quotient in the control and breakfast experiments was practically at the basal level immediately before the various exercise periods; in the sugar experiments it rose in one instance as high as 0.92, or 0.15 above the basal.

One hour after a high carbohydrate breakfast, there was no change in muscular efficiency although the resting respiratory quotient immediately before exercise had risen considerably.

Muscular efficiency, therefore, was not improved by taking food nor was it correlated with the height of the respiratory quotient at rest immediately before exercise. Accepting the usual interpretation of the respiratory quotient, it may be concluded that muscular efficiency was not related to the carbohydrate oxidation prevailing at the time exercise was begun.

REFERENCES

BACHMANN, G. AND J. HALDI. J. Nutrition **13:** 157, 1937a.
This Journal **120:** 579, 1937b.

CARPENTER, T. M. AND E. L. FOX. Arbeitsphysiol. **4:** 570, 1931a.
Ibid. **4:** 527, 1931b.

HAGGARD, H. W. AND L. A. GREENBERG. Diet and physical efficiency. Yale University Press, New Haven, 1935.

THE INFLUENCE OF BILE ON THE MOTILITY OF THE DOG'S COLON

E. A. GALAPEAUX, R. D. TEMPLETON AND E. L. BORKON[1]

From the Departments of Physiology of the University of Chicago and Loyola University School of Medicine

Received for publication July 21, 1937

The use of bile as a constituent of suppositories is referred to in the writings of Hippocrates (1); however, its use in enemas is seldom mentioned in clinical literature. The oral administration of bile or bile salts is in common practice for relief in constipation. The idea that bile stimulates peristalsis is commonly taught and is supported by some investigators and textbooks (2, 3, 4). On the contrary, various investigators (5, 6, 7) have reported a depression in the motility of excised strips taken from both the small and large intestine. These latter observations were confirmed by Nakata (8) when the serous coat of the strip was exposed to the action of bile but when the mucosal coat was exposed even to a much stronger concentration of bile the effect was slight or absent.

In previous work (9, 10) our findings have not supported the common view that the efficiency of an enema as a colon cleanser is dependent on the extent to which it augments intestinal activity. White mineral oil was found to be much more efficient for stimulating muscular activity of the colon than water or soap solution; however, the latter types of enemas were more efficient than oil in eliciting defecation.

Hummon and Templeton (11) have observed fluoroscopically in four human subjects that the expulsion of barium enemas in some patients was not associated with visible activity of the colon while in others peristaltic activity in the transverse colon seemed to be essential to evacuation. In the two cases where no visible activity appeared during voluntary evacuation the subjects had just been diagnosed as normal by the fluoroscopic study. In the two subjects where voluntary effort alone was not sufficient to empty the colon, the fluoroscopic examination had just revealed adhesions in one case at the splenic flexure and in the other adhesions at both the splenic and hepatic flexures which limited the usual mobility of the colon at these points.

In the light of these previous observations it seems impossible to deduct from the efficiency of a substance as an enema its effect on intestinal

[1] Henry Strong Denison Fellow.

activity. The common use of bile as a corrective in constipation makes it desirable to know its specific effect on colon motility.

For this study we used four cecostomized dogs which had been under laboratory conditions and used in other colon studies for over a year. The animals were trained to lie quietly on a table for a period of 400 minutes or more. Two tandem balloon systems (12) of three balloons each modified for correlating proximal and distal activity (13) were used for recording intestinal motility (14, 15). One system (proximal system) was inserted by way of the cecostomy. To the other (distal system) a rubber tube was attached between the middle and terminal balloons for intracolonic injections without disturbing the animal while the tracing was being made. This system was inserted by way of the anus. Dog's gall bladder bile was selected as the type to be used in order to obviate the possibility of a difference in composition of the bile of different species being responsible for the action elicited.

The maximum quantity of gall-bladder bile to be used in these experiments was taken as that quantity equal to the largest amount of tap water which could be injected into the colon without causing an appreciable effect on the motility. Thus controlled the volume increase in the content of the colon caused by the injection could be disregarded.

In preliminary studies it was observed that significant, consistent changes in colon activity seldom followed the injection of 20 cc. of tap water. This quantity, therefore, was taken as a maximum for intraluminal injections and 25 control experiments were conducted in which after 200 minutes of motility had been recorded 20 cc. of tap water were injected without disturbing the animal or breaking the continuity of the tracing. In order to further obviate the possibility of distention being a factor only 10 cc. were injected at a time and 15 minutes were permitted to intervene before the second injection. The tracings were then continued for an additional 200 minutes. To control smaller injections of bile and to compare with 20 cc. injections of water 27 other experiments were conducted in which only 10 cc. of water were injected in two 5 cc. quantities after the first 200 minutes of each tracing had been obtained.

In tabulating the results of these experiments only the quantity of motility as measured by the time in which a segment or segments were active was considered. A segment was considered active if a contraction or contractions lasted for one minute or more. It was considered quiet if two or more minutes appeared in which there was no activity. Thus the activity for each segment for each 50 minute period was determined and from the sum of the activity in the six segments the average activity per segment was calculated.

In those experiments in which 20 cc. of water were injected the average activity per segment (table 1) in the 200 minute control period varied

from about 24 to 26 minutes per 50 minute period. Following the injection of water the average activity was found to vary from approximately 24 to 27 minutes per 50 minute period. The injection of this quantity of water had no appreciable effect on the average activity. The average activity for the control period preceding the injection of 10 cc. of water varied from about 26 to 28 minutes per 50 minute period. Following the injection of this quantity of water the average activity varied from about 26 to 29 minutes per 50 minute period. The injection of this quantity of water after the 200 minute control period did not seem to have a significant effect.

Since the quantity of activity in the 200 minute control period varies from day to day in the same animal and especially in different animals, the question arose concerning what influence the degree of activity which a colon manifests at the time of the injection might have in determining

TABLE 1

Average minutes of activity per 50 minute periods

	CONTROL PERIOD				FOLLOWING INTRACOLONIC INJECTIONS			
	50 minute periods							
	1	2	3	4	5	6	7	8
Control experiments, injection of 20 cc. of water	24.9	26.2	24.7	24.1	24.5	25.5	25.1	27.1
Control experiments, injection of 10 cc. of water	26.1	27.7	27.3	26.6	29.5	29.9	27.4	28.7
Injection of 20 cc. of bile	29.2	27.0	25.3	29.2	15.7	14.7	21.1	20.9
Injection of 10 cc. of bile	24.9	24.3	24.3	27.1	15.4	15.1	17.2	22.8

the type of response elicited. To answer this question the experiments in which 20 cc. of water had been injected were divided into two groups (fig. 1, part A). In the first group were 11 experiments in which the average activity in the first 200 minutes was 50 per cent or more per 50 minute period. In the second group were 14 experiments in which the average activity in the first 200 minutes was less than 50 per cent per 50 minute period. The average per cent of activity in the first group varied from 65 to 61 during the control period. A decline in the average per cent of activity was observed to have begun 150 minutes after the beginning of the experiment. This decline continued at a slightly augmented rate following the injection of water for the next 50 minutes after which a gradual rise to approximately the starting level of activity was observed. In the second group the average per cent of activity varied from 37 to 48 during the first 200 minutes. A small augmentation in the increasing activity was observed following the injection of 20 cc. of water

which is exactly opposite to the results observed in the first group. Since in a large number of experiments approximately the same number would occur in each group an adequate standard is obtained for the study of intracolonic injections of other substances.

Those experiments in which 10 cc. of water were injected were also divided into two groups (fig. 1, part B). In the first group there were 14

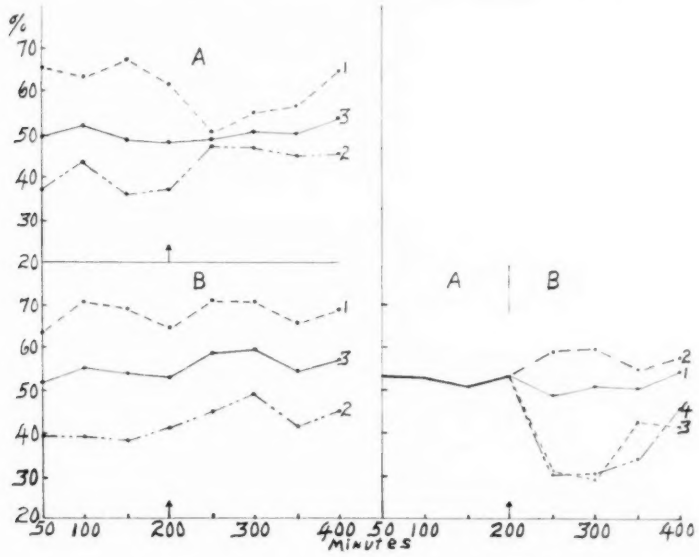


Fig. 1

Fig. 2

Fig. 1. Represents the average per cent of colon activity per segment. Part A represents the per cent of activity in experiments where 20 cc. of water were injected. Curve 1, experiments in which the activity was above 50 per cent during the first 200 minutes (control period); curve 2, experiments in which activity was below 50 per cent during the first 200 minutes; curve 3, average of 1 2. ↑ = injection of 20 cc. of water into colon. Part B represents same as part A except that 10 cc. of water were injected.

Fig. 2. Represents average colon activity per segment. Part A. Control period (104 tracings). Part B. After injection of bile or water. Curve *1*—Effect of 20 cc. of water (25 expts.). Curve *2*—Effect of 10 cc. of water (27 expts.). Curve *3*—Effect of 20 cc. of bile (24 expts.). Curve *4*—Effect of 10 cc. of bile (28 expts.).

tracings in which the activity in the control period was 50 per cent or more per 50 minute period. In the second group there were 13 tracings in which the activity in the control period was less than 50 per cent per 50 minute period. Following the injection of 10 cc. of water there was a slight but probably insignificant increase in the activity of both groups.

The grand average for both groups gave an increase in activity from 53 to 59 per cent 100 minutes later which is probably within the range of experimental variation.

In those experiments in which bile was injected the same animals were used and the same technique was employed as in the control experiments. In 24 experiments 20 cc. of bile were injected in two 10 cc. portions 15 minutes apart after a control period of 200 minutes. In 28 experiments in which only 10 cc. of bile were injected, two 5 cc. portions were administered 15 minutes apart at the end of the control period.

A total of 104 experiments (fig. 2) were conducted in which the first 200 minutes of each served as a control period. The average activity for this control period varied from 50 to 53 per cent per 50 minute period. At the close of this control period those experiments in which 20 cc. of

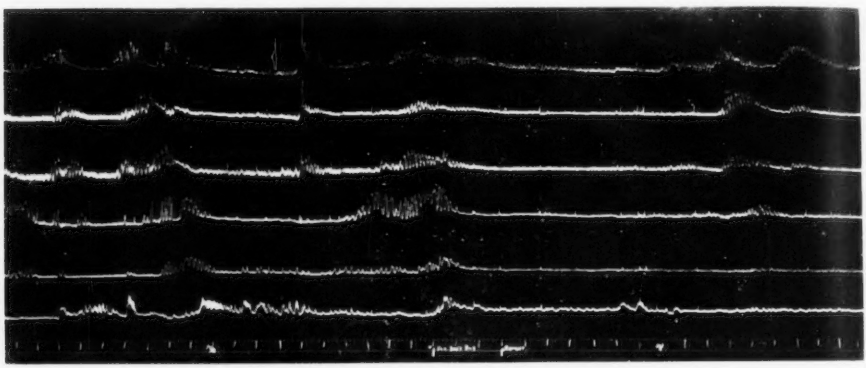


Fig. 3. Six balloon tracings of colon motility. ↑ = Injection of 10 cc. of bile in two 5 cc. portions.

water were injected gave an average activity in the succeeding 200 minutes varying from 49 to 54 per cent. Those experiments in which 10 cc. of water were injected gave an average activity varying from 59 to 55 per cent. Following the injection of 20 cc. of bile the average per cent of activity varied from 30 to 41. The average activity following the injection of only 10 cc. of bile varied from 30 to 45 per cent. The depression of activity following the injection of either of the above quantities of bile was usually manifested within a few minutes after the first injection. The degree of depression was marked in the first 50 to 100 minutes (fig. 3) returning slowly to approximate that of the control period 150 to 200 minutes later.

In all cases where bile was injected defecation followed within a few minutes after the end of the experiment and in most cases a marked

tenesmus was manifested. Considering tenesmus as an indication of a higher degree of efficiency in eliciting defecation, bile in 10 and 20 cc. quantities is more effective than 100 or 200 cc. quantities of white mineral oil, water or 1.25 per cent soap solution. The efficiency of these substances in eliciting defecation is inversely related to their ability to augment activity when injected into the colon.

The evidence obtained concerning the relation between circular colon activity and defecation is not sufficient to justify conclusions concerning the mechanism involved. It may be suggested that the inhibition of the types of activity recorded by the tandem balloon systems may be more frequently associated with the urge and act of defecation than an augmentation of such activity. Having received an urge to defecate the depression of activity may leave a more relaxed tube through which voluntary effort can more easily propel the material.

From another viewpoint we may consider that under our experimental conditions two mechanisms existed which might conflict: first, the effect on colon activity and, second, an inhibition of the defecation urge. The direct effect on colon activity may be an augmentation in some cases inhibited because of an excessively strong defecation urge elicited while the animals are in a posture unnatural for the defecation act. On the other hand if the urge is not too strong the substance injected into the colon may elicit an increased activity and thus explain the observations made in the study on oil enemas (9).

SUMMARY

1. In a series of experiments in which the manifest control activity of the colon was approximately 50 per cent, the intracolonic injection of 10 and 20 cc. quantities of tap water in divided quantities 15 minutes apart had no appreciable effect.

2. The injection of 10 and 20 cc. quantities of dog's gall-bladder bile into the colon was followed by a marked depression in colon activity lasting from 50 to 100 minutes.

3. In all cases where bile was injected into the colon defecation followed within a few minutes after the experiment and a marked tenesmus was usually manifested.

4. Two possible theories as to the relation of a depressed colon activity to defecation are suggested.

REFERENCES

(1) Hippocrates (from J. F. Montague). Med. Rec. **139**: 91, 1934.
(2) Schuepbach. Ztschr. Biol. **51**: 1, 1910.
(3) Best and Taylor. The physiological basis of medical practice. Baltimore, 1937.
(4) Bodansky. Introduction to physiological chemistry. New York, 1934.

(5) Ott, I. and J. C. Scott. Proc. Soc. Exper. Biol. and Med. **6:** 13, 1908.
(6) d'Errico, G. Biochem. Centralbl. **10:** 647, 1910.
(7) Schwarz and Magerl. Pflüger's Arch. **202:** 509, 1924.
(8) Nakata, H. Mitt. a. d. Med. Akad. zu Kioto **7:** 722, 1933.
(9) Borkon, E. L. and R. D. Templeton. This Journal **118:** 775, 1937.
(10) Templeton, R. D. and E. L. Borkon. Unpublished data.
(11) Hummon and Templeton. Unpublished observations.
(12) Templeton, R. D. and W. F. Bollens. J. Lab. and Clin. Med. **15:** 585, 1930.
(13) Templeton, R. D. and H. Lawson. This Journal **96:** 667, 1931.
(14) Templeton, R. D. and H. Lawson. J. Lab. and Clin. Med. **17:** 1244, 1932.
(15) Templeton, et al. This Journal **116:** 153, 1936.

THE EXPERIMENTAL PRODUCTION OF SEVERE HOMOGENEOUS OSTEOPOROSIS BY GASTRECTOMY IN PUPPIES

R. A. BUSSABARGER,[1] SMITH FREEMAN AND A. C. IVY

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago, Illinois

Received for publication July 22, 1937

The gastric juice is generally considered to be a factor in the absorption of calcium and phosphorus from the intestine. This is because the acid of the gastric juice renders dietary calcium salts more soluble and assists in the maintenance of the acidity of the intestinal content, which favors the absorption of calcium and phosphorus (1). That the *stomach plays an essential rôle* in the development of bone in growing puppies, and probably children, is demonstrated by the results to be recorded in this paper. In fact, the only way by which the importance of the stomach for the adequate absorption and retention of bone building materials can be determined is by removing the stomach in growing animals receiving an adequate diet (2).

Five puppies of an age ranging from 103 to 172 days were gastrectomized for the purpose of studying the production of erythrocytes. Two puppies were kept as controls. The puppies were fed a mixed diet (stock diet) consisting of lean ground parboiled meat, bread, a prepared dog food and one pint of milk daily. They consumed about 2 lbs. of food a day. The available calcium in the diet was 1 gram per day. The ratio of Ca:P was about 0.5:1.0. In addition the animals received daily two teaspoonfuls of U.S.P. cod liver oil. Blood studies consisting of red blood cell counts, hemoglobin and hematocrit determinations, and measurements of red blood cell diameters were made at frequent intervals.

Because of the spontaneous occurrence of two pathologic fractures in puppy 7, which occurred 71 days after operation, and because of the subsequent appearance of progressive bone deformities in all the gastrectomized animals, x-ray, blood chemistry and bone ash studies were made.

After an initial weight retardation during the first post-operative month, the weight curves of the gastrectomized animals were comparable to those of the controls. However, the weights of the gastrectomized animals

[1] Jessie Horton Koessler Fellow of the Institute of Medicine of Chicago.

became constant at a slightly lower level than those of the controls. The gastrectomized animals had a mild, hypochromic, oligocythemic, normocytic anemia, which disappeared spontaneously when their body weights became constant about 220 days after operation.

From four to six weeks post-operatively the gastrectomized puppies generally manifested a "leg-weakness" with flattening of the feet. The legs, particularly the front legs, became bowed in from four to eight weeks. The photograph of one of the gastrectomized animals is shown in figure 1. The picture was taken 510 days after operation, the dog being 103 days old when the gastrectomy was performed. The picture does not reveal the marked genu valgum of the hind legs. The roentgenogram of the foreleg of the dog is shown in figure 2 alongside the film of the foreleg of

Fig. 1

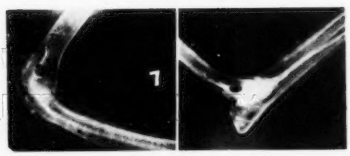


Fig. 2

Fig. 1. Photograph of a gastrectomized puppy (no. 7) 510 days after operation which was performed at 103 days of age.

Fig. 2. The roentgenogram of puppy 7, figure 1, alongside that of the control, taken 407 days post-operatively when both animals were 510 days old.

the control dog. The film was made 407 days after operation when both animals were 510 days old. The decreased radio-density, the thin cortex and wide medulla of the gastrectomized animal is apparent. No evidence of rickets was observed in any of the roentgenograms made of these animals.

The serum calcium, phosphorus, and phosphatase of the gastrectomized puppies were usually within the normal range (table 1).

The tibia was removed from one of the gastrectomized animals and from the control litter-mate for study. The tibia of the gastrectomized animal weighed only 60 per cent of that of the control. The per cent ash was 95 per cent of that of the control, and the per cent calcium contained in the ash was identical in both. This result showed, as might be expected, the presence of deficient ossification.

When calcium lactate (0.5 gram per kilogram dissolved in water) was given orally to the gastrectomized animals, a rapid rise in serum calcium occurred; only the usual slight change occurred in the control animals (table 2). This is because the stomach of the control animals slowly ejected the calcium into the intestine, whereas in the gastrectomized animals all the calcium was immediately available for absorption. This result (table 2) showed that the intestine of the gastrectomized animal can absorb soluble calcium salts.

TABLE 1

Showing the serum calcium, inorganic phosphorus and phosphatase values of normal and gastrectomized dogs at various ages

ANIMALS	AGE	NUMBER of ANIMALS	NUMBER OF DETERMINATIONS	SERUM		
				Calcium	Phosphorus	Phosphatase
	days			*mgm. per cent*	*mgm. per cent*	*units*
Gastrectomized puppy	70 to	10	29	12 0	6 62	5 98
Normal puppy	220	4	9	12 0	7 92	3 84
Gastrectomized puppy	220 to	11	14	11 9	6 34	4 68
Normal puppy	370	3	4	12 3	5 82	3 99
Gastrectomized puppy	370 to	7	8	11 4	4 63	2 95
Normal puppy	570	3	3	11 0	4 77	1 54
Gastrectomized puppy	570 to	3	16	10 4		
Normal puppy	970	2	8	10 7		
Gastrectomized puppy	970 to	3	3	10 5	3 25	
Normal puppy	1070	2	2	11 1	4 12	
Range of variation:						
Gastrectomized puppy		13	70	7 6 14 7	3 15 9 80	1 35 9 70
Normal puppy		5	26	8 6 13 7	3 72 8 50	1 00 5 17

When calcium was injected intravenously it was removed from the blood stream at the same rate in the controls and gastrectomized animals. This result shows that the rapid rise in blood calcium noted above was not due to a disturbed rate of removal of calcium from the blood stream (table 2).

The study was then extended to include a group of 20 puppies. Fourteen were gastrectomized; a pouch of the entire stomach with an esophagoduodenal anastomosis was made on three; and 3 litter-mates were retained as controls. The ages at which the operation for gastrectomy was performed varied: 42 days, 4; 43 days, 3; 63 days, 5; 122 days, 2. The ages

at which the operation for an entire stomach pouch was performed were: 62 days, 2; 63 days, 1.

TABLE 2

Showing the results of blood calcium studies

PROCEDURE	ANIMALS	NUMBER OF DOGS	NUMBER OF TESTS	AVERAGE SERUM Ca (MGM. PER CENT) AND RANGE OF VARIATION					
				Fasting	0.5 hour	1 hour	1.5 hours	2 hours	3 hours
$CaCl_2$ intravenously; 0.2 cc. of 12 per cent kilogram body weight	Gastrectomized	7	9	11 2 8 3-12 6	12 0 10 5-13 0	11 3 9 1-12 4	11 2 8 6-12 6	11 3 8 4-12 6	11 4 9 0-12 8
	Normal	3	4	11 5 9 7-13 6	12 6 12 2-13 6	11 9 11 2-13 8	12 2 10 9-13 0	11 8 10 4-13 8	11 8 10 0-13 4
					1 hour	2 hours	3 hours	4 hours	5 hours
Ca lactate by gastric tube 0.5 gram per kilogram	Gastrectomized	3	6	10 4 9 5-11 6	15 8 11 2-24 6	13 9 10 2-20 2	12 2 9 6-17 2	11 3 8 8-15 0	10 5 8 5-13 3
	Normal	4	5	11 5 10 6-12 6	12 0 10 9-13 0	11 9 11 1-12 7	11 5 10 4-12 4	11 5 10 0-12 5	11 0 10 0-12 3
Ca lactate injected into jejunum 0.5 gram per kilogram	Exteriorized jejunal loop normal dogs	3	3	11 5 11 3-11 6	12 4 12 0-12 8	12 3 11 7-12 7	12 1 11 8-12 4	11 7 10 9-12 5	11 5 11 1-12 2
Fed meal containing milk, meat and cereal	Gastrectomized	4	5	10 9 9 9-12 2	12 6 11 2-14 6	11 5 11 2-12 4	11 2 10 6-12 2		
	Normal	3	3	11 9 11 3-12 7	10 8 10 0-12 5	11 0 10 2-12 4	11 1 10 0-12 8		
Fed meal without milk, but with water	Gastrectomized	3	4	10 5 9 5-11 0	9 8 8 9-10 4	9 8 8 4-10 6	9 7 8 6-10 4		
	Normal	2	3	10 7 10 0-11 2	9 2 8 9-10 0	10 0 9 6-10 4	9 9 9 7-10 0		
Fed meal of boiled beef heart with water	Gastrectomized	6	10	9 1	8 5		8 3		
	Normal	3	4	9 4	9 0		9 1		
500 cc. milk by gastric tube	Gastrectomized	1	1	11 8	16 5	15 8	13 7		
	Normal	1	1	10 4	10 6	10 5	10 4		
500 cc. milk injected into jejunum	Exteriorized jejunal loop-normal dogs	2	2	11 4	11 2	11 4	11 2		

To be noted: That the serum Ca of the gastrectomized puppy is sometimes lower than normal, that oral milk and Ca cause a greater increase in serum Ca than in the normal, that a meal of beef, only, which stimulates pancreatic secretion well tends to decrease serum Ca, and that comparing the data on the jejunal administration of milk and Ca with that on the gastrectomized animals definitely indicates that the intestine of the latter animals is different from the normals. This latter difference is explained as due to the intestine of gastrectomized animals having become adapted to some extent to receiving and retaining substances not coming slowly from the stomach.

Eight of the gastrectomized puppies were grouped as a series to study the effect of the addition of calcium salts to the diet. Three were given calcium gluconate; 2, calcium lactate; 2, calcium diphosphate; and 1, no additional calcium. The calcium salts were added to the diet outlined above and administered in amounts which varied with the age of the animal. Equivalent amounts of calcium were added in each case; from operation to 90 days of age, 218 mgm. of Ca daily; from 90 days to 180

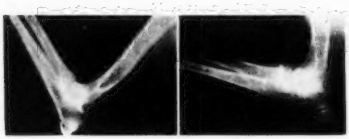


Fig. 3

Fig. 4

Fig. 3. Roentgenogram of puppy 11, the control (left), alongside that of gastrectomized puppy 12, which received calcium diphosphate. One hundred and thirty days later the bone ash on humerus of no. 12 was 50.5 per cent (shaft) and that on no. 11 was 54.3 per cent.

Fig. 4. Roentgenogram of puppy 12 showing one of the spontaneous fractures. At 152 days after the spontaneous fractures, one had healed, the other had not.

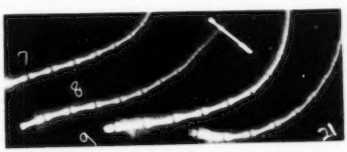


Fig. 5. Showing the difference in the calcification of the bones of the tail in litter mates. The tail labeled 9 is the control, nos. 7 and 8 received calcium gluconate as a complement and no. 21 calcium diphosphate. The bone ash on no. 9 was 53.0 per cent; on no. 7, 46.2 per cent; on no. 8, 43.6 per cent; on no. 21, 37.4 per cent.

days of age, 436 mgm. Ca daily; from 180 days onward, 650 mgm. Ca daily. Viosterol (5,000 units) was given once weekly in addition to the cod liver oil.

The addition of soluble calcium salts in the amounts given does not completely prevent the appearance of bone deformity and deficient calcification, although it is of definite benefit. The addition of calcium diphosphate has no apparent benefit. Figures 3, 4 and 5 illustrate

these points. The differences in the degree of calcification between the gastrectomized and control puppies are manifest in the bones of the tail (fig. 5; note the bone ash values).

What is the cause of the marked deficiency of calcification of the bones in the growing gastrectomized dog? We have shown that soluble calcium salts can be absorbed; and repeated determinations of serum Ca, P, and phosphatase show these constituents to be normal in most instances. Two possible etiological factors remain. One is that food calcium is inadequately absorbed. The other is that after the calcium is absorbed, it is inadequately retained. Both of these possibilities may operate.

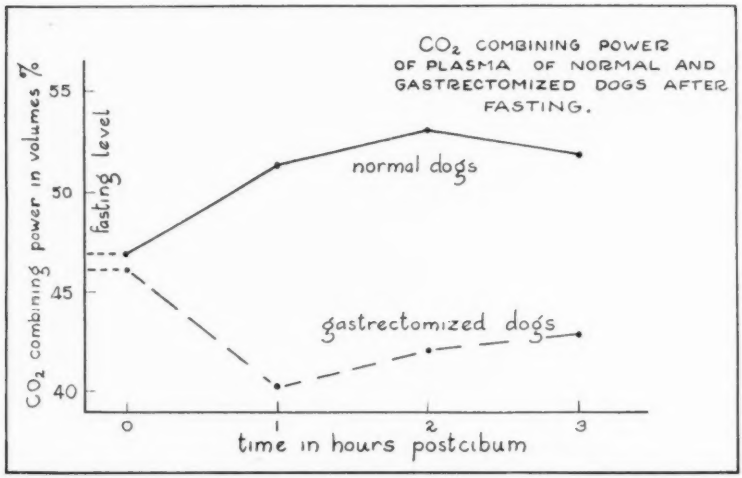


Fig. 6. A chart showing the occurrence of a decrease in plasma CO_2 combining power of the blood after a meal in gastrectomized dogs.

According to Albright, Bauer and Aub (3), acidosis may serve to mobilize calcium from the bones. And the gastrectomized animal may show a postcibal acidosis on secreting pancreatic juice and bile in response to a meal. Accordingly the CO_2 combining power of the plasma (whole blood also in one experiment) was determined after feeding. The serum calcium was also determined in these experiments.

The data on the CO_2 combining power of the plasma (fig. 6) show that after feeding an acidosis occurred for a period of three hours, which was as long as it was followed (4). (It is to be kept in mind that gastrectomized animals eat slowly.) The controls showed a slight increase in the alkaline reserve of the blood. The serum calcium of the gastrectomized dogs fell slightly after eating, except when milk was added, then

it rose an average of 2 mgm. per cent. The control dogs showed a slight decrease in serum calcium after a mixed meal (table 2).

Thus, the postcibal acidosis which occurs in these achlorhydric animals must be considered as a factor which operates to decrease calcium deposition in the bones. But this cannot be the only factor concerned because the same degree of diminished calcification of the bones and deformity occurs in puppies with a pouch of the entire stomach, in which

TABLE 3

Calcium metabolism

Three litter-mates at 215 days of age. (Figures represent gram totals of each five day period.)

ANIMAL	5 DAY PERIOD	FOOD INTAKE 0.052 gram Ca per 100 grams	CALCIUM ELEMENT IN FOOD INGESTED	CALCIUM ADDED	TOTAL CALCIUM INTAKE	TOTAL CALCIUM OUTPUT URINE AND FECES	CALCIUM RETAINED
Puppy 23, gastrectomized 151 days	1	2287	1 190	0	1 190	1 25	−0 060
	2	2280	1 186	0	1 186	1 26	−0 074
	3	2297	1 194	*0 905	2 099	1 69	+0 409
	4	3488	1 814	0	1 814	1 91	−0 096
	5	2250	1 170	†0 130	1 300	1 36	−0 060
Puppy 27 gastrectomized 151 days	1	2280	1 186	0	1 186	1 39	−0 204
	2	2287	1 189	0	1 189	1 36	−0 171
	3	2291	1 191	*0 905	2 096	1 97	+0 126
	4	3350	1 742	0	1 742	2 19	−0 448
	5	2278	1 185	†0 130	1 315	1 34	−0 025
Puppy 28, normal control	1	2148	1 117	0	1 117	0 869	+0 248
	2	2280	1 186	0	1 186	0 817	+0 369
	3	2292	1 192	*0 905	2 097	0 672	+1 425
	4	3410	1 773	0	1 773	1 579	+0 194
	5	2257	1 174	†0 130	1 304	1 120	+0 184

* Two grams calcium gluconate added to food daily.

† Three and four-tenths cubic centimeters of 10 per cent calcium gluconate intravenously daily.

the stomach secretes acid but the acid drains to the exterior. It should be pointed out, however, that since such pouches are devoid of vagal innervation, the secretion of acid is hyponormal.

In regard to the second possible factor, it is rather obvious that gastric achylia and the relatively rapid intestinal transport of food materials through the intestine would predispose to inadequate absorption of calcium, particularly food calcium and the less soluble calcium salts.

Consequently calcium balance experiments were made. In these ex-

periments specially constructed cages were used. Two litter-mate gastrectomized puppies and one control puppy were used for five five-day calcium metabolic periods. Other similar groups have also been studied.

It was found that the gastrectomized puppies manifested a negative calcium balance while on a diet of meat and cereal. The normal control maintained a positive calcium balance. When two grams of calcium gluconate (0.181 gram Ca) were added to the diet of meat and cereal fed the gastrectomized puppies, a positive balance was maintained (table 3). On a diet similar to the stock diet (this diet contained less milk; the

TABLE 4

Calcium balance studies

Litter-mates

ANIMAL NUMBER		DIET FIVE TEST PERIOD	FOOD AND CALCIUM INTAKE FOR 5-DAY PERIOD		Ca* INTRAVENOUSLY TOTAL 5 DAYS	TOTAL Ca INTAKE 5 DAYS	CALCIUM OUTPUT IN URINE AND FECES 5 DAYS				TOTAL Ca OUTPUT 5 DAYS	Ca RETAINED	PER CENT RETAINED
							Urine		Feces				
			Food	Ca			Volume	Ca	Weight	Ca			
			grams	*grams*	*mgm.*	*grams*	*cc.*	*grams*	*grams*	*grams*	*grams*	*grams*	
23	Total gastric pouch 222 days	Mixed meal, with milk	6587	2 668		2 668	2386	0 041	316	2 220	2 261	0 407	15 6
		Mixed meal, no milk	3291	1 711	0 465	2 176	1615	0 023	258	1 320	1 343	0 833	38 3
26	Gastrectomy 222 days	Mixed meal, no milk	1885	0 980	0 465	1 445	710	0 014	197	1 240	1 254	0 191	13 2
27	Gastrectomy 222 days	Mixed meal, with milk	4729	1 915		1 915	2360	†	195	†	1 822	0 093	10 1
		Mixed meal, no milk	2676	1 392	0 465	1 857	1675	0 016	221	1 590	1 606	0 251	13 5
28	Normal control	Mixed meal, no milk	2002	1 041	0 465	1 506	720	0 042	95	0 680	0 722	0 784	52

* Ten cubic centimeters of 10 per cent Ca gluconate (93 mgm. Ca) daily.

† Feces and urine combined because of soft feces.

calcium content was only 40.5 mgm. per 100 grams of food as fed), the gastrectomized and total pouch puppies maintained a positive calcium balance (table 4). Intravenously administered calcium gluconate (0.026 gram of Ca element per day) while on the meat and cereal diet, failed to produce a positive balance (table 3). However, when this amount was increased to 0.093 gram of Ca element per day, a positive calcium balance was established (table 4). It is interesting to note that in the total pouch puppy the per cent of the calcium administered that was retained was greater than that retained by the two gastrectomized puppies. This

lends further support to the conception of acidosis as a factor in preventing proper retention of absorbed calcium by the gastrectomized puppies even though no increased excretion of urinary calcium was noted (table 4).

These results show that it should be possible to maintain gastrectomized puppies on a positive calcium balance provided adequate quantities of soluble calcium salts are administered, and support the observations noted above on the alleviating effects of soluble calcium salts on the bony deformities. Whether the bony deformities may be entirely alleviated by administering soluble calcium salts orally in amounts larger than those used, remains to be demonstrated.

Other incidental observations. All of the gastrectomized dogs have a normal lens. A few of the animals gastrectomized at an early age manifest enamel hypoplasia. The oldest "puppy" in our series has some hyperplasia of the parathyroids as determined grossly and histologically; the younger puppies, as far as we have determined, have normal parathyroids. Tetany, muscular and nervous irritability, although examined for, were not observed. Eight dogs we have on hand, which were gastrectomized at an adult age, have bones which are less radio-opaque and have thinner cortices than other adult dogs maintained in excellent condition on the same diet including cod liver oil; they do not manifest bony deformities, however (fig. 7).

Discussion. It seems to be apparent from our studies that three, or perhaps four, factors are concerned in the failure of the bones to calcify normally in gastrectomized puppies. The relative importance of these three factors cannot be evaluated with the evidence at hand. Some unknown factor may also be involved.

The first factor to be considered is the importance of the absence of HCl. Although it has been recognized that gastric juice is important in the solution of calcium salts, it has been thought to be a dispensable factor in calcium absorption. It has been frequently pointed out that anacid patients do not suffer from calcium deficiency; but, such statements have been based on experience with adults and not growing children. Givens and Mendel (5) have found that the addition of acid or base to a diet does not significantly affect calcium balance, but that the addition of HCl increases the output of urinary calcium in dogs. Zucker (6) has found that the addition of acid or alkali did not affect the total calcium balance, acid leading to a shift of calcium and phosphorus from feces to urine, the reverse effect being obtained with sodium bicarbonate. Essentially similar findings have been reported by others (7). But because of the effect of the administered acids and alkalies on gastric and pancreatic secretion and their effect on body chemistry, these experiments are not directly pertinent to the question of the effect of gastric anacidity on calcium metabolism.

It is well known that in gastric anacidity the reaction of the upper intestine is less acid. This would tend to diminish the absorption of calcium, since it is generally believed that most of the calcium is absorbed in the upper intestine before the acid of the gastric juice has been completely neutralized (1). In the absence of HCl, organic acids, such as carbonic and lactic acid and possibly the bile acids, assist in the absorption of calcium. In this connection it would be of obvious interest to study the effect of vitamin D on the absorption of calcium and phosphorus in gastrectomized dogs, because it has been shown that the administration of vitamin D in rickets causes the feces to become less alkaline (1).

The second factor to be considered is the absence of the reservoir function of the stomach. In the absence of the stomach the food is rather

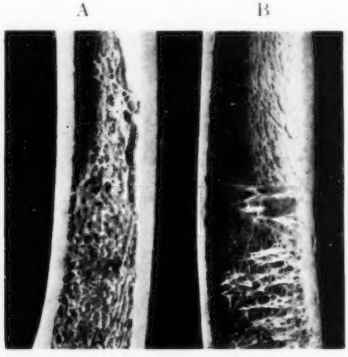


Fig. 7. Showing the difference between the bones of a normal (A) control dog and one (B) gastrectomized at 3 years of age and living nine years post-operatively. Note that the bone of the gastrectomized dog has a wider medullary space, thinner cortex and fewer trabeculae.

rapidly moved through the upper part of the intestine, and is incompletely digested. Gastrectomized puppies and dogs generally tend to compensate for lack of the reservoir function of the stomach by eating slowly; and, about twice the amount of a normal maintenance diet is required and ingested. The more rapid rate of passage of food through the upper intestine, where it is believed (1) that most of the calcium is absorbed and the incompleteness of the digestion of food while there, should decrease the degree of calcium absorption.

The third factor to be considered is the postcibal acidosis which occurs generally in gastrectomized animals. That this factor must be considered is shown by the observations of Farquharson, Salter, Tibbetts and Aub (3) and by their review of the literature. An increase in the acid eliminated in the urine and particularly an increase in urinary am-

monia is associated with an increase in calcium and phosphorus elimination in the urine. The "acid tide" manifested by the gastrectomized animals should be expected on the basis of the observations in the literature (8).

The deficient calcification of the bone in our gastrectomized puppies is quite analogous to the simple or homogeneous osteoporosis observed in children with celiac disease without rickets. Parsons (9) states that a fracture may be the symptom that first brings the child with celiac disease to the physician, and that the whole bone is fragile, osteoporotic, with a thin cortex and an open and delicate trabecular meshwork. It is generally believed that the underlying cause of the bone changes in celiac disease not complicated with vitamin D deficiency is deficient calcium and phosphorus absorption. It is known that serum calcium and inorganic phosphorus may be normal, as in our dogs, in celiac disease, although when the disease is present in children under 2.5 years of age, both mineral elements, especially calcium, are lower than normal, which was occasionally observed in our animals.

In relation to our observations, A. F. Hess (9, 10) has pointed out that it is not certain whether only one variety of osteoporosis exists. For example, it has not been shown that the osteoporosis produced by an acid diet is the same as that caused by a deficient absorption of calcium, or that caused by mild hyperparathyroidism. The use of the gastrectomized puppy should help to elucidate this question, as well as the question whether osteoporosis due to deficient calcium absorption predisposes to rachitic changes due to vitamin D deficiency.

In a recent article Aub, Tibbetts and McLean (11) submitted evidence indicating that some "unknown factor" markedly increased calcium absorption from the intestine in a growing boy. On the basis of our present knowledge of the digestive activities of the stomach of the growing child, the "unknown factor" cannot be said to be resident in the stomach; further study may show that it is.

The most obvious difference between a gastrectomized subject and a subject with complete gastric anacidity is that the former is devoid of the reservoir function of the stomach and the latter is not.

Perhaps a fourth factor is concerned. Gastrectomized dogs manifest a hyperplasia of the bone marrow, which may contribute to the production of osteoporosis.

SUMMARY AND CONCLUSIONS

These observations show that the stomach is essential for the normal growth and development of the bony skeleton, because when the stomach is removed from growing puppies the bones do not ossify to a normal extent in the presence of a diet adequate for normal puppies. The disturbance in ossification of the bones or the homogeneous osteoporosis is

so severe that bony deformities and even spontaneous fractures result. The osteoporosis is analogous to that observed clinically in severe cases of "celiac disease" without rickets. The deficient ossification of the bones is apparently due to a combination of three factors, namely, *a*, the absence of HCl which normally renders the less soluble calcium salts more soluble and assists in the maintenance of an acid reaction in the intestine; *b*, the absence of the reservoir function of the stomach which increases the speed of intestinal transport of food substances, and *c*, the presence of a postcibal acidosis ("acid tide") which tends to decrease calcium retention. Other factors may be involved.

REFERENCES

(1) Stewart and Percival. Physiol. Rev. **8:** 283, 1928.
Kay. Physiol. Rev. **12:** 384, 1932.
Schmidt and Greenberg. Physiol. Rev. **15:** 297, 1935.
Bills. Physiol. Rev. **15:** 1, 1935.
McGowan. Biochemistry J. **26:** 113, 1932; **27:** 934, 943, 1933.
Steenbock, Hart, Sell and Jones. J. Biol. Chem. **56:** 375, 1923.
(2) Bussabarger, Lederer and Ivy. This Journal **116:** 22, 1936.
Lederer and Crandall. This Journal **118:** 52, 1937.
(3) Albright, Bauer and Aub. J. Clin. Invest. **10:** 187, 1931.
Farquharson, Salter, Tibbetts and Aub. J. Clin. Invest. **10:** 221, 1931.
(4) Bussabarger, Bradley and Lederer. Proc. Am. Physiol. Soc., 1937.
(5) Givens and Mendel. J. Biol. Chem. **31:** 421, 1917.
Givens. J. Biol. Chem. **35:** 241, 1918.
(6) Zucker. Proc. Soc. Exper. Biol. Med. **18:** 272, 1921.
(7) Goss and Schmidt. J. Biol. Chem. **86:** 417, 1930.
Mussehl, Hill, Blish and Ackerson. J. Agric. Res. **42:** 115, 1931.
(8) Dodds. J. Physiol. **54:** 342, 1921; Brit. J. Exper. Path. **4:** 13, 1923.
Dodds and Smith. J. Physiol. **58:** 157, 1924.
Kanders and Porges. Wien. Arch. Klin. Med. **5:** 379, 1923.
Hubbard and Munnford. Arch. Int. Med. **35:** 576, 1925; J. A. M. A. **87:** 922, 1926.
Gamble and M'Iver. J. Exper. Med. **48:** 849, 859, 1928.
(9) Parsons. Am. J. Dis. Child. **43:** 1293, 1932.
(10) Hess. Rickets, including osteomalacia and tetany. Philadelphia, 1929.
(11) Aub, Tibbetts and McLean. J. Nutrition **13:** 635, 1937.

A QUANTITATIVE STUDY OF THE EFFECTS OF ACETYLCHOLINE AND ADRENALIN ON THE NICTITATING MEMBRANE

R. S. MORISON AND G. H. ACHESON

From the Department of Physiology in the Harvard Medical School

Received for publication July 22, 1937

Great interest attaches to the properties of adrenalin and acetylcholine because of their close relation to the excitation process in autonomic effectors (Cannon and Rosenblueth, 1937). In most instances, these drugs have been found to have on a given organ qualitatively antagonistic effects. Thus, acting on the heart, acetylcholine slows and adrenalin accelerates the rate; and acting on the cat's non-pregnant uterus acetylcholine causes contraction while adrenalin causes relaxation. It is striking, therefore, that both substances bring about qualitatively similar contractions of the nictitating membrane (n. m.) of the cat (Rosenblueth, 1932a).

Clark (1927) and Rosenblueth (1932b), using one or another of the drugs on various effectors, have obtained fairly precise quantitative relationships between dose and response which fit simple hyperbolic equations. These observations have been used to support the hypothesis that the drugs act upon cells by combining in a monomolecular equilibrium with specific cell receptors. Relatively little information is available as to the nature of such receptors. The question arises, would different drugs having the same effect on the cell employ the same receptor or different receptors?

The present study was undertaken in order to illuminate this question by comparing quantitatively the action of acetylcholine and adrenalin on the same effector (n. m.). None but qualitative studies have been made of the effects of acetylcholine on the n. m. and no quantitative comparisons of the actions of acetylcholine and adrenalin on any other effector are available. The relation between dose of acetylcholine and response of the n. m. was therefore determined and compared quantitatively with the same relation for adrenalin. The mode in which the responses to these two agents sum, when they are given simultaneously, was also studied.

METHOD. Cats were used under dial anesthesia (Ciba, 0.7 to 0.8 cc. per kilogram). The n. m. was sensitized to the agents used, by aseptic removal of the superior cervical ganglion one to five weeks before the acute experiments. Its contractions were recorded on a kymograph by a thread passed over a pulley to an isotonic lever giving a magnification of approximately 15 times. The drugs used were adrenalin (Parke, Davis) and

crystalline acetylcholine hydrochloride (Merck), the latter made up in concentration of 1:10 and used for about a week. On the day of the experiments appropriate dilutions were made with either tap water or Ringer's solution so that the volume of each injection was constant, usually 0.1 cc. In order to keep the injections as small as possible, to minimize effects on blood pressure, the injections were made into the ipsilateral carotid artery. The adrenal glands were removed in all the experiments.

The concentration-action curves were tested for their correspondence to rectangular hyperbolas of the general form $y = \frac{x}{k' - kx}$ or $k' - kx = \frac{x}{y}$ by plotting x against $\frac{x}{y}$, a procedure which yields a straight line if the curves are hyperbolic. The horizontal asymptotes of the hyperbolas are then equal to the reciprocals of the slopes of the straight lines obtained.

RESULTS.. *A. Concentration-action curve of acetylcholine.* The curve relating dose of acetylcholine to response of the n. m. proved to approxi-

TABLE 1

Asymptotes in centimeters for each concentration-action curve, calculated as the reciprocal of the slope of the straight line passing through the experimental points when plotted as dose/response against dose

DATE	ADRENALIN	ACETYLCHOLINE	ADRENALIN AND ACETYLCHOLINE
January 22	5.0 to 6.7		6.0
April 16	6.2	6.3	
May 5	6.4	6.0 to 6.9	6.2
May 7	6.2 to 7.0	5.1	6.8
May 12	6.5 to 7.0	7.7	8.0
May 21	7.5 to 8.3		7.5
June 5	7.5	6.9	

mate a right hyperbola as has been shown for adrenalin on the n. m. by Rosenblueth (1932b) and for acetylcholine on other organs by Clark (1933). Its asymptote agreed, within the limits of experimental error, with that for the adrenalin curve in the same experiment (table 1, columns 1 and 2). Larger doses (5 to 60 times by weight in different experiments) of acetylcholine than of adrenalin were necessary for equal effects, but a linear adjustment of the abscissae rendered the curves superimposable in all instances. A typical example is illustrated in figure 1.

B. Responses to mixtures of adrenalin and acetylcholine. In some experiments the response to a dose of adrenalin (a_1) was given in combination, first, with an additional dose of adrenalin (a_2), and, second, with a dose of acetylcholine (c_2), such that if given alone the response to c_2 was equal to that produced by a_2. The resulting summed response was the same in

both cases. This leads to the conclusion that acetylcholine sums with adrenalin just as the single substances sum with themselves.

In most of the experiments a method of more general application but similar in result was followed. The concentration-action curves of adrenalin and acetylcholine were obtained and plotted as in figure 1. The

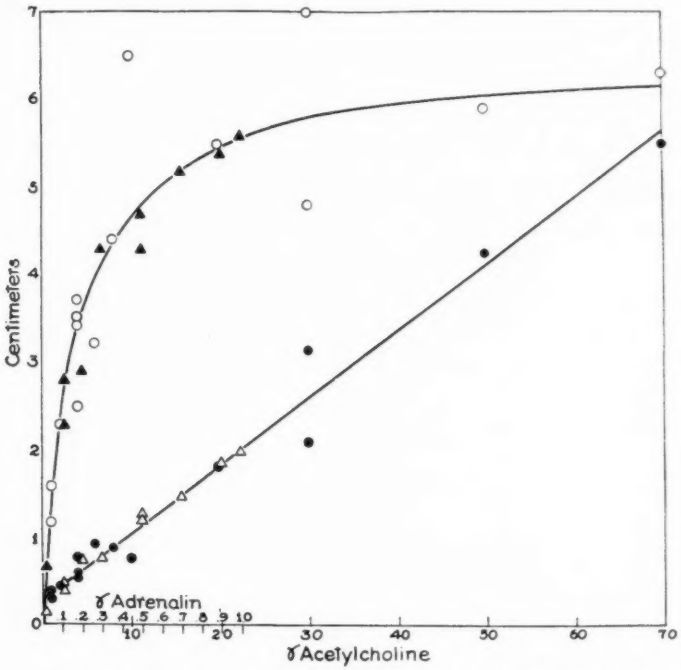


Fig. 1. Upper curve: concentration-action curves of adrenalin and acetylcholine on the nictitating membrane. Ordinates: response in centimeters. Abscissae: gammata of adrenalin and gammata of acetylcholine injected into the ipsilateral carotid artery. Circles: responses to acetylcholine. Solid triangles: responses to adrenalin. Lower curve: the same points plotted as dose/response against dose. Ordinates: dose/2 times response. The dose here was read on the acetylcholine scale for both adrenalin and acetylcholine. Abscissae: as in upper curve. Dots: acetylcholine points. Open triangles: adrenalin points.

responses to mixtures of acetylcholine and adrenalin were then plotted, the abscissa of a particular point being determined by adding the dose of adrenalin on the adrenalin scale to the dose of acetylcholine on the acetylcholine scale. A series of points representing the summation of the two substances could then be plotted and the general shape of the curve and its correspondence to the original curves could be determined (fig. 2).

In figure 2 a second curve was constructed on the assumption that each of the substances, when given in combination, could exert its full effect uninfluenced by the presence of the other, i.e., that they lead to contraction through completely independent processes. Here the abscissae were the same as those employed above, but the ordinates represented the linear

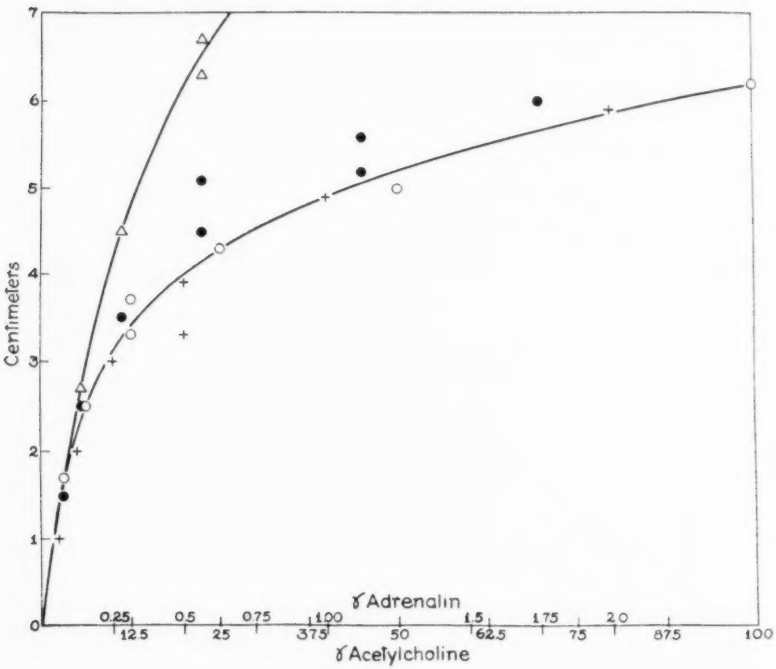


Fig. 2. Ordinates: responses in centimeters. Abscissae: gammata of adrenalin and gammata of acetylcholine injected into the ipsilateral carotid artery. Lower curve: concentration-action curves of adrenalin and acetylcholine on the nictitating membrane. Circles: responses to acetylcholine. Plus marks: responses to adrenalin. Upper curve (triangles): expected curve if response to mixture of adrenalin and acetylcholine were the linear sum of response to adrenalin plus response to acetylcholine. Dots: actual responses to mixtures of adrenalin and acetylcholine. Abscissae for dots and corresponding triangles in gammata of adrenalin and of acetylcholine, respectively: 0.03 and 1.5; 0.06 and 3.1; 0.125 and 6.2; 0.25 and 12.5; 0.5 and 25; 0.5 and 50.

sum of the responses to the two components of the mixture given separately. Figure 2 shows that the summation followed more nearly the hyperbolic than the linear course. As may be seen from table 1, column 3, the correspondence of the asymptotes of all three hyperbolas in all the completed experiments is striking.

The responses to combinations of the two drugs have been compared with the two types of summation represented in figure 2, and the average percentage deviations in each experiment are shown in table 2. Since the maximal ability of the contractile mechanism of the n. m. may not be much greater than the response produced by a maximal dose of adrenalin, summed responses which would have been above the level, if summation were linear, have not been included in the table. Table 2 also demonstrates that the summed effect of the two substances is not linear but more nearly hyperbolic.

DISCUSSION. It has been known for some time that the responses of the nictitating membrane to acetylcholine and adrenalin are qualitatively similar (Rosenblueth, 1932a). The present observations indicate that they are quantitatively similar as well. Thus, the two substances yield concentration-action curves of the same general shape and apparently iden-

TABLE 2

Summary of the average per cent error from linear and from hyperbolic type of addition calculated from curves as in figure 2, by experiments

See text for explanation

DATE	OBSERVATIONS	PER CENT ERROR (AVERAGE)	
		Linear	Hyperbolic
January 22	6	−29.6	−1.4
May 5	3	−18.1	+8.8
May 12	4	−17.6	+6.9
May 21	15	−30.0	+0.5
June 5	4	−26.0	−2.0
5 experiments	32 observations	−24.2	+2.2

tical asymptotes (p. 150). Furthermore, the summation of responses to the two substances given in combination shows a close relation in their mode of action. Doses of either acetylcholine or adrenalin, which alone give identical effects, may be added to a standard dose of either of them to produce the same summed response. In other words, the response of a partially contracted n. m. to an injection of either of the drugs is the same in height whether the partial contraction was originally brought about by adrenalin or by acetylcholine.

Rather higher concentrations of acetylcholine than of adrenalin (5 to 60 times in these experiments) are necessary to elicit equal responses, but this may be at least partially explained by the fact that acetylcholine is destroyed much more rapidly in the body and so reaches the membrane in reduced concentration.

The simplest way to account for the qualitative and quantitative similarities between the actions of acetylcholine and adrenalin would be to

postulate the local liberation of one of the substances by the other, the final curve of summation being due to the action of the liberated substance. Such an explanation is, however, at odds with the fact that pyrogallol potentiates and prolongs the responses to adrenalin but not those to acetylcholine (Bacq, 1935), whereas eserine potentiates and atropine depresses the responses to acetylcholine much more than these agents affect the action of adrenalin.

It becomes of interest, therefore, to consider the data in relation to the current conception of the mode of action of these drugs. Clark (1927, 1933) and Rosenblueth (1932b) attribute the hyperbolic shape of the concentration-action curves to a monomolecular reversible reaction between the drug and a hypothetical receptor in the activated cell, according to the law of mass action. Its horizontal or upper asymptote, representing the maximal possible response, is then dependent upon the amount of receptor present. The finding of similar asymptotes for the curves produced by different drugs may then be taken as evidence that identical amounts of their respective receptors are present in the reacting structures, or that the same receptor is available to both substances. Further evidence for the latter conclusion is derived from the study of the summation produced by the drugs in combination with each other. If one accepts the preceding explanation of the concentration-action curves of the single drugs, the fact that acetylcholine sums with adrenalin in the same way that it sums with itself (p. 151) shows that a dose of either drug is capable of quantitatively reducing the amount of receptor available for combination with a succeeding dose. It follows, therefore, that both drugs act by combining with a common receptor.

When this work was undertaken it was thought probable that owing to the rather different chemical nature of the two substances they would combine with different receptors. In this case, there being no competition for a common receptor, combinations of the two drugs should have resulted in lineally summed responses. Such a result would have helped to establish the important assumption that the effect of a combined drug-receptor complex on the contractile mechanism is lineally proportional to its concentration, and would have helped to place the hyperbolic step in the chain of reactions leading to contraction early in the excitation process. A similar analysis of the summed responses of sympathin on the nictitating membrane had previously been performed (Rosenblueth and Morison, 1934) which, although requiring the elaboration of a few subassumptions, supported the view.

Although the present results are consistent with the current interpretation of the shape of the concentration-action curves if the two drugs are assumed to combine with the same receptor, there are certain objections to this view. In the first place, there is some evidence (Clark and Raven-

tos, 1937) that atropine antagonizes the effects of acetylcholine by combining with its receptor and preventing the latter substance from doing so. These workers have also shown that if two drugs act through a single receptor, atropine should antagonize both equally; and they give evidence that closely related compounds, acetylcholine and Me_4N, for instance, are similarly affected by atropine. In the nictitating membrane, however, atropine depresses the response to acetylcholine much more than it does that to adrenalin.

It is also of interest to consider the situation which would exist in the heart if the drugs in question act by combining in a mass-action equilibrium with receptors. Acetylcholine slows the heart over a range of doses of from 0.1 to 425 γ (Clark and White, 1927), which is approximately that which causes increasing contraction of the n. m. Adrenalin yields excitatory effects on both organs and the receptor is probably the same in both (cf. Cannon and Rosenblueth, 1937). Since acetylcholine can combine with this excitatory receptor the slowing which is actually recorded with acetylcholine should be due to the production of a large excess of inhibitory over excitatory complex. This could happen if the inhibitory receptor was present in great excess, but the concentration-action curve of acetylcholine would then be the expression of a complex interaction between the inhibitory and excitatory compounds. On these grounds the interpretation of the concentration-action curve in any simple manner would not be warranted.

The present evidence is not inconsistent with the view that drugs act by combining with receptors in the activated cells; it merely emphasizes a few of the difficulties which may beset any attempt to attribute the shape of concentration-action curves to such a specific step in a very complex series of reactions leading to the response of irritable tissues (cf. Roepke, 1937).

SUMMARY

The responses of the nictitating membrane of the cat to various doses of adrenalin and of acetylcholine were studied.

The concentration-action curves of the two substances were found to be hyperbolas of the same general shape. Their horizontal asymptotes, representing the maximal possible response to either substance were also identical (fig. 1, table 1).

Simultaneous application of the two drugs yielded responses which were the hyperbolic and not the linear sum of the responses to the separate constituents (fig. 2 and table 2), i.e., adrenalin summed with acetylcholine in the same way that each substance summed with itself.

The implications of these results are discussed and are found to raise difficulties to the view that the shape of the concentration-action curves is

due solely to a monomolecular equilibrium reaction between the drugs and a cell receptor.

REFERENCES

Bacq, Z. M. Arch. Internat. Physiol. **42:** 240, 1935.

Cannon, W. B. and A. Rosenblueth. Autonomic neuro-effector systems. New York, 1937.

Clark, A. J. J. Physiol. **61:** 530, 1927.

The action of drugs on cells. Baltimore, 1933.

Clark, A. J. and J. Raventos. Quart. J. Exper. Physiol. **26:** 375, 1937.

Clark, A. J. and A. C. White. J. Physiol. **64:** 11P, 1927.

Roepke, M. H. J. Pharmacol. Exper. Therap. **59:** 264, 1937.

Rosenbleuth, A. This Journal **100:** 443, 1932a.

Ibid. **101:** 149, 1932b.

Rosenblueth, A. and R. S. Morison. Ibid. **109:** 209, 1934.

A STUDY OF THE MECHANISM WHEREBY COITUS EXCITES THE OVULATION-PRODUCING ACTIVITY OF THE RABBIT'S PITUITARY[1]

CHANDLER McCUSKEY BROOKS

From the Department of Physiology, Johns Hopkins University School of Medicine

Received for publication July 23, 1937

Ovulation normally occurs in the rabbit only as a result of coitus or intense sexual excitement (Hammond, 1925; Brooks, 1937). The work of Fee and Parkes (1929), Smith and White (1931), McPhail, Parkes and White (1933) and others has led to the conclusion that at the time of coitus and for a brief period thereafter the anterior lobe of the pituitary gland liberates a substance or substances which by direct action on the ovary (Friedman, 1929) produce ovulation. This pituitary activity must be initiated, in some manner, by the act of coitus or the coincident emotional excitement. Brambell and Parkes (1932) and Bellerby (1934), among others, have suggested that on coitus the anterior lobe of the pituitary is excited reflexly. Most certainly the association of the glandular discharge with mating does indicate that an excitatory mechanism exists. The major aim of this research was to ascertain whether this pituitary activity is humorally or neurally mediated.

If the act of coitus and the associated emotional excitement cause a discharge of the anterior lobe of the pituitary gland by initiating nervous impulses which pass to the gland over one or more nerve pathways connecting the central nervous system and the anterior lobe, severance of these nerve fibers should prevent the occurrence of ovulation after coitus. If, on the other hand, the pituitary activity is dependent on some chemical excitant transported to it from the nervous system or from an intermediate endocrine gland, denervation of the pituitary should not prevent the induction of ovulation by coitus provided the denervation is performed without damage to the blood supply of the gland. Complete denervation of the pituitary gland was attempted in an effort to decide which of these two possible mechanisms of excitation is the one essential to the coitus-induced activation of the anterior lobe.

[1] A preliminary report of this work has been published in the Proc. Am. Physiol. Soc., This Journal **113:** 18, 1935 and **119:** 280, 1937.

This work was aided by a grant (to Dr. Philip Bard) from the Committee for Research in Problems of Sex, National Research Council.

METHODS. The only known nervous pathways to the pituitary gland are fibers from the superior cervical ganglia and the fibers passing to it from the hypothalamus by way of the pituitary stalk. In a few cases both superior cervical ganglia and the cervical sympathetic trunks were removed. It has been reported by Fletcher (1898) and by Feldberg (1926) that in the rabbit a few fibers pass to the ears and possibly to other regions of the head from the stellate ganglia by paths other than the cervical sympathetics. To eliminate these, the stellates, as well as the cervical sympathetic nerves and the superior cervical ganglia, were removed. This operation was performed with some difficulty and the chain was usually broken under the first rib. In three operations particular care was taken to avoid this break and the possibility of leaving a few nerve fibers and cells in that region. Both superior cervical ganglia with chains attached were dissected free from connections and thrust down into the chest cavity of their respective sides. The chest was then opened bilaterally and the chains pulled down into the thorax; the stellates and entire thoracic chains were freed and removed unbroken. These operations most certainly eliminated the possibility that any neural connections between the thoraco-lumbar preganglionic outflow and the pituitary escaped interruption. Finally, in six additional rabbits the removal of abdominal sympathetic chains was performed after the animals had recovered from preliminary cervical and thoracic sympathectomies. A regeneration of the nerves probably could not have occurred but the animals mated within two to three weeks and these studies were completed before sufficient time had elapsed to permit any extensive regeneration.

A sub-temporal approach to the pituitary stalk was generally employed, but in two instances an orbital approach was used. In the temporal approach the bone over both hemispheres was removed, the rabbit turned on its side, the dura incised and the brain gently pushed up and to one side; the pituitary stalk could then be seen and severed. The stalk was always cut as close to the gland as possible but usually the section was such that much cerebrospinal fluid escaped through the lumen of the cut stalk. This flow of fluid and the slight bleeding which occurred obscured the field and unless a complete transection was made at the first attempt the connecting remnants could not easily be seen and severed. After the stalk was cut the brain was carefully reoriented and the wound closed by subcutaneous and skin sutures.

The cranial operations performed in the elucidation of another problem of a kindred nature (Brooks, 1937) served as a partial control for these operations. In three additional cases the cranium was opened and the stalk exposed but not severed. These animals mated and ovulated in a completely normal manner after a few days.

Pentobarbital sodium (0.7 grain per kilogram of body weight) was the

anesthetic employed for all intracranial operations. It was injected intraperitoneally. If an animal was not fully anesthetized by this dose the pentobarbital sodium was supplemented by ether. Ether alone was used for sympathectomies. Animals recovered quickly from these cranial operations and from the sympathectomies and soon became indistinguishable from normal rabbits in external appearances and general behavior. The other procedures followed in the study of these preparations will be mentioned in connection with a description of the results obtained.

Results. *Pituitary activation after sympathectomies.* Each of five female rabbits from which the stellate and superior cervical ganglia of both sides had been completely removed ovulated normally following coitus. Three animals in which the superior cervical ganglion, cervical sympathetic nerve, stellate ganglion and thoracic chain of each side were removed intact likewise ovulated on mating. Five additional female rabbits from which both sympathetic chains had been completely removed not only repeatedly ovulated but also successfully bore and reared litters of normal size. In the female rabbit a sympathetic nerve supply to the hypophysis is, therefore, not essential to its gonadotropic function nor is a sympathetic innervation of a mammary gland necessary for its lactation.

Berkley (1895), Dandy (1913), Pines (1926), Hair (1937) and others have demonstrated that nerve fibers of sympathetic origin do reach the anterior lobe of the pituitary in the species which they studied. There is some evidence that these sympathetic fibers which originate chiefly in the superior cervical ganglia, may exert some influence on the gonadotropic function of the pituitary. In the case of the rabbit Friedgood and Pincus (1935) have reported that long continued electrical stimulation of the cervical sympathetic nerves induces maturation of eggs in unruptured ripe follicles. In three additional cases ovulation and the beginning of luteinization of the follicles were found. This evidence indicates that the sympathetic innervation of the anterior lobe may be capable of influencing the activity of the gland but the long period of stimulation necessary to produce the slight signs of activity observed suggests that in the intact animal the sympathetics are of minor importance in this connection. The experiments performed in the course of the present investigation and those of Hinsey and Markee (1933 b) adequately prove that if a reflex stimulation of the anterior lobe of the pituitary acting through efferent nerve fibers is responsible for the initiation of its coitus-induced activity some nerve pathway other than these sympathetic fibers must be involved.

The question of non-sympathetic nerve fibers to the anterior pituitary. There is abundant evidence in the literature that nerve fibers arising in the hypothalamus pass down the pituitary stalk to the pars posterior and pars intermedia (Penfield, 1932; Roussy and Mosinger, 1933; Fisher, Ingram and Ranson, 1935). Cajal (1894), Tello (1911) and Hair (1937)

are among those who have claimed that, at least in certain mammals, a few of these fibers cross into the anterior lobe. Croll (1928) found such fibers in the anterior lobe of the rabbit. An attempt was made to confirm the work of Croll and to ascertain whether or not these anterior lobe fibers could be found after removal of the sympathetic nerve supply. A variety of slightly differing silver-impregnation methods was used. In two normal and one sympathectomized rabbit Cajal's technique was employed. Ranson's (1911) modification of Cajal's method was most commonly followed and it was used in the study of the pituitaries of four normal and three sympathectomized rabbits. Cuajunco's modification of Agduhr's Bielschowsky technique was used in two instances with relatively unsatisfactory results. In three additional cases Bodian's (1936) protein-silver method was employed. From one rabbit of this latter group of animals the superior cervical and stellate ganglia had been removed. All the animals spoken of as sympathectomized or with superior cervical and stellate ganglia removed had been operated on from two to six weeks before the histological studies were made. This is a sufficiently long period of time to permit complete degeneration of sympathetic fibers.

With all these techniques and in all these preparations fibers resembling nerve fibers were found in the anterior lobe of the pituitary. The fibers were not extremely numerous and seemed to vary in quantity in the different animals but they were found in all parts of the lobe. In several instances fibers were seen which were entering the anterior lobe from the pars intermedia. Fibers were always more numerous in the parts of the anterior lobe adjacent to the pars intermedia. They were not invariably associated with blood vessels but occasionally they did join small vessels for a part of their course. The fibers were long and could be followed through several serial sections as they coursed among the cells. In a few cases they appeared to terminate in knob-like or button-like endings on or in close proximity to a cell wall. Thus the findings of Croll are confirmed. In addition, it is shown that these fibers are not of sympathetic origin since they are present after removal of the ganglia which supply all sympathetic postganglionic fibers to the tissues of the head and neck. Because of the irregularity of the path taken by these fibers in their passage among the anterior lobe cells difficulty was encountered in obtaining convincing photographs. Figure 1-E, however, gives some idea of the appearance of these fibers.

Although the histological evidence for the presence of nerve fibers of hypothalamic origin in the anterior lobe is not overwhelmingly convincing it is sufficient to suggest that the stalk should be transected in attempts to denervate the gland. The fibers which are stained are not numerous and it may be thought that this fact argues against their functional significance. The number of fibers necessary to control a given quantity of glandular

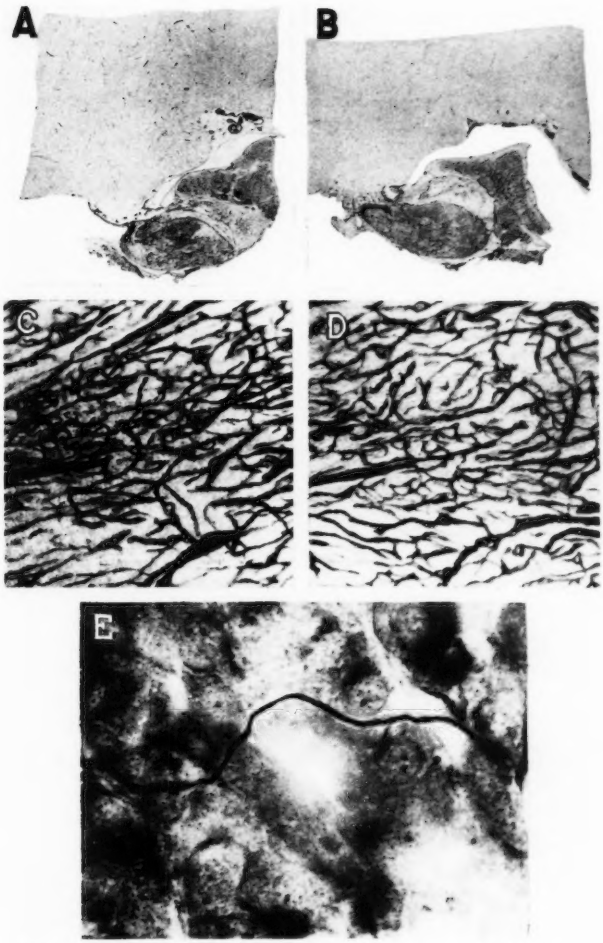


Fig. 1. A. India ink injection of the pituitary of a normal rabbit.

B. India ink injection of the pituitary from an animal with complete section of the stalk. The larger number of blood vessels visible in the brain tissue of the normal rabbit indicates that that individual was more successfully injected. The difference in the vascularity of the two pituitaries is, therefore, not as great as it appears to be. Portions of decalcified bone can be seen partially surrounding both glands.

C. Higher magnification (approx. × 100) of a portion of the anterior lobe of the injected normal gland.

D. Higher magnification (approx. × 100) of a similar portion of the gland from the stalk-cut rabbit. The vessels are equally numerous but those of the normal gland contain more ink.

E. Portion of an anterior lobe fiber in the pituitary of a sympathectomized rabbit (magnification approx. × 1480).

tissue is not known. Since it is generally recognized that epithelial tissues are difficult to stain this justifies the assumption that there may be many more fibers present in the anterior lobe than are revealed by the technique used.

Persistence of mating and absence of the associated ovulatory response after complete section of the pituitary stalk. Sixteen rabbits with severed pituitary stalks failed to ovulate though they mated frequently. Some of these animals were kept for over a year and studied carefully and frequently during that time. Others were kept for a month or more and given ample opportunity to mate. Each animal was tested daily for at least one month of its post-operative survival period. The mating records show a recurrence of mating periods at fairly regular intervals. Since these periods varied in different individuals one hesitates to describe this as a mating cycle. Table 1 shows the mating history of a few stalk-cut animals. One of the several reasons for not regarding this periodicity of receptivity as cyclical behavior is that the unwillingness of these females to mate at certain times was most certainly due to the manner in which the male made his approach. Occasionally a female would receive one male but not another. Certain males had a higher degree of success with particular females than did others. Unquestionably sexual receptivity is an emotional reaction to some extent and can be modified by factors other than the condition of activity of the female endocrine glands. Therefore, a failure to mate does not invariably indicate a state of anestrus.

Vaginal smears were taken from many of the animals during the mating test-periods. The smears were obtained with a small pipette from which a fraction of a cubic centimeter of saline was first injected into the vagina. Although changes in cell types were observed the smears failed to reveal clearly-defined cyclical changes. They did show quite clearly, however, that during a long period of sexual inactivity the smears contained chiefly leucocytes and a few epithelial cells of regular outline with large nuclei. Such smears (fig. 2-A) almost without exception were correlated with a state of non-receptivity. During periods of receptivity when the animals mated frequently the smears were composed almost entirely of cornified cells of irregular shape with small pycnotic nuclei (fig. 2-B). A high percentage of cornified cells did not invariably indicate receptivity but it can be said that mating animals generally yielded a smear in which cornified cells predominated.

Ten of the sixteen rabbits with complete stalk sections possessed intact sympathetic systems. Under the conditions of these experiments the sympathetic nerves were incapable of mediating ovulation. This is an additional indication that the sympathetic nerves to the anterior lobe normally play a minor rôle, if any, in the regulation of the gonadotropic function of the gland. In the remaining six rabbits the stellates or su-

TABLE 1

RABBIT NUMBER	STATE	DAYS AFTER OPERATION	RESULTS OF DAILY MATINGS†
18	Stalk cut	90	– – –

perior cervical ganglia were removed to see if this operation produced any change in activity or in the histology of the pituitary. No changes were detected.

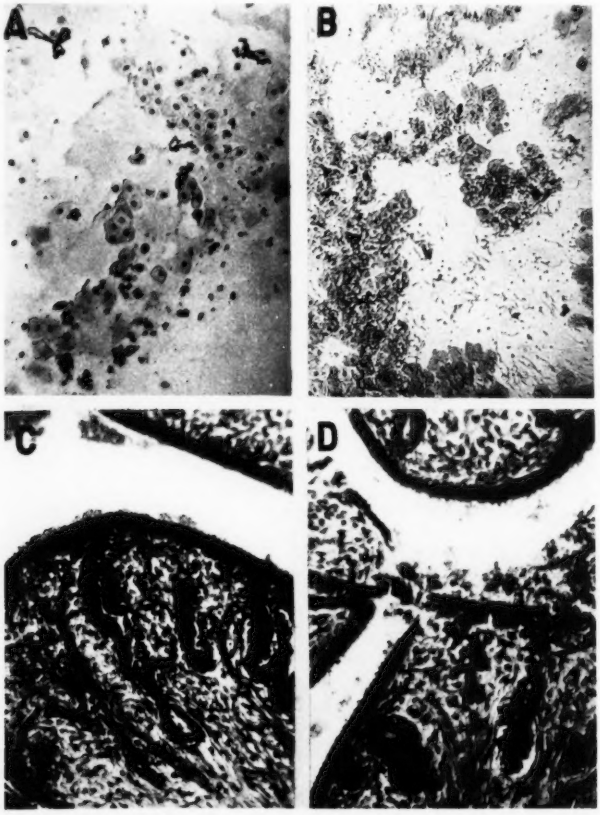


Fig. 2. A. Vaginal smear characteristic of stalk-cut rabbits during periods when they refuse to mate.

B. Smear characteristic of stalk-cut animals during receptive periods. These two smears were taken from the same animal. The rabbit mated several times on the day that smear B was taken.

C. Fragment of uterus from a normal rabbit.

D. Similar portion of the uterus of a rabbit whose pituitary stalk had been severed three months previously.

The pituitaries and adjacent regions of the hypothalamus of five rabbits whose pituitary stalks had been severed were prepared for histological

study by silver impregnation techniques. The Ranson pyridine-silver method was used in three cases and Bodian's technique in two. Two preparations yielded inconclusive results because the hypothalamic fibers were not clearly and uniformly differentiated and therefore the apparent absence of fibers in the pituitary was without significance. In three cases the nervous tissue was satisfactorily stained and the nerve fibers were clearly differentiated. In these pituitaries there was a complete absence of the fibers seen in the glands of normal animals. Usually a few scattered fibers were present in the upper part of the stalk just below the point of transection but in the pars anterior, and lower part of the pars posterior no fibers could be found.

At operation it was sometimes difficult to see the stalk clearly and it was usually impossible to determine by mere inspection whether or not the transection was complete. Histological examinations were, therefore, always made by cutting and studying serial sections of the pituitary and overlying hypothalamus. In a few preparations after fixation of the tissues the surrounding bone was gently cut away from the pituitary but in the majority of cases the entire head was injected with fixing fluids; part of the hypothalamus and the adjacent pituitary, still enclosed in the sella turcica, were removed; the bone was decalcified and the entire block sectioned serially. Thus the possibility of breaking stalk remnants at the time of autopsy was avoided. This procedure necessitated a series of control experiments on normal animals to see whether or not decalcification interfered with the histological techniques employed. It was found that decalcification did not affect nerve fibers stained by the Cajal or Ranson pyridine-silver method. Therefore, when silver techniques were employed the entire block of tissue was stained, then decalcified and sectioned. It was likewise found that decalcification did not destroy the peculiar affinities of various cells and cell structures for the stains used. The eosinophils and basophils of these decalcified preparations had practically the same appearance as did those of glands which had not been exposed to the hydrochloric acid. Serial sections were cut in the longitudinal axis parallel to the sagittal plane. Scar tissue found at the point of transection constituted a non-functional anatomical connection between the stalk remnant attached to the brain and the pituitary. Great care was necessary in following through the series to make sure that no contiguity of functional tissue remained. This point was emphasized by the study of seven animals in which it was thought that attempts to sever the stalk had been successful. These animals all eventually ovulated as a result of mating but it was found when the pituitary tissues were sectioned that the stalks had not been completely cut. In the case of three of these animals the intact connecting stalk remnant was difficult to find because it had been so displaced that the connection could not be seen in any one section of the series.

The animals with partially transected stalks were of interest for several reasons. In four of them stalk injury was not great and they ovulated after the first or second postoperative mating. The three animals in which the stalk section was not quite complete did not ovulate as a result of the usual periodic matings. Rabbit 17 was almost continuously receptive but failed to ovulate until mated a great number of times within a few hours (87 times in 7 hours; see table 1). Following that experience she did ovulate but just two corpora lutea were formed although other apparently normal ripe follicles were present in the ovaries. The other two rabbits behaved in a similar manner (one ovulated after 12 briefly spaced matings, the other after 22). Possibly any one of the three would have ovulated with fewer matings; at any rate coitus once or twice a day was ineffective. These animals required greater stimulation. The cervical sympathetics and superior cervical ganglia were removed from five of the seven rabbits with partially cut stalks but ovulation still occurred following mating. In three animals (nos. 17, 28 and 29) Ranson's pyridine-silver technique was used and in one (no. 107) Bodian's method; in all four the characteristic fibers were found in the pars intermedia and pars anterior. Fibers were scarce in the pituitary of no. 17, but a few were definitely seen. The diminished number of fibers, the fragmentary stalk connection and the abnormal intensity of stimulation necessary for the production of ovulation suggest that the pathway for the stimuli which normally reach the pituitary and induce its ovulation-producing activity had been almost completely interrupted.

These nerve-like fibers were not found in the five rabbits with stalks completely cut which were studied by the silver methods. This fact most certainly suggests that the fibers are responsible for the post-coital activation of the anterior lobe. However, such a conclusion concerning the importance of this supposed anterior lobe nerve supply cannot be accepted if other pituitary abnormalities result from stalk section.

The effect of stalk-section on the blood supply of the pituitary. The venous plexus of the stalk was unavoidably damaged when the stalk was severed. Thus a circulatory abnormality was created. It is entirely possible that the portal system which carries blood from the hypothalamus to the pituitary (Wislocki and King, 1936) is essential for the functional normality of the gland. An effort was made to judge the degree of vascular abnormality in the glands of animals with completely and partially transected stalks. Carmine gelatin and india ink injections (made at pressures between 140 and 180 mm. Hg) did reveal slight circulatory abnormalities in both types of preparation. Difficulty was encountered, however, in obtaining complete injection of all vessels even in the normal animal and though identical procedures of injection were followed in all cases a uniform degree of success was not obtained. At best these techniques furnish only

a rough indication of the state of the circulation in an organ. Precise conclusions concerning comparative degrees of circulatory deficiency cannot be obtained in this way. A study of numerous injected preparations (6 normal animals, 4 animals with partially and 10 with completely transected stalks) did permit certain general conclusions. The blood supply of the gland is rendered slightly abnormal by partial or complete transection of the stalk. In all the animals which had been operated on the portal system either had not been completely destroyed by the operation or had been reëstablished in part. As revealed by the injections, the circulatory abnormalities in two of the animals which had partially transected stalks and had ovulated in response to coitus were fully as great as those seen in many of the preparations with completely severed stalks. In three animals, whose stalks had been completely cut, filling of the blood vessels by the injection mass appeared to be practically as complete as in normal rabbits. Figure 1 shows portions of the injected anterior lobes of a normal and a stalk-cut rabbit.

The effect of stalk section on the cells of the anterior pituitary. Histological studies were made of the pituitaries of four animals with partially transected stalks and of a series of glands from twelve animals which had been killed at various times after their pituitary stalks had been cut. These were compared with sections of glands from normal rabbits. The glands were fixed in formol-Zenker and sectioned serially at 5μ. Five different stains were used in this study: aniline acid fuchsin differentiated with picric acid or with methyl green; hematoxylin-eosin; Mallory "triple"; and a slightly modified form of the orange-G and aniline blue combination of Mallory II stain used alone. Each was employed in the staining of certain sections from each gland.

The pituitaries of animals which had been killed 30 to 291 days after stalk transection showed easily detectable abnormalities in the pars posterior, pars intermedia and pars anterior. These were, however, never as extensive as those observed by Mahoney and Sheehan (1936) after stalk section in the dog.

The pars neuralis was shrunken and somewhat fibrosed. The changes observed were similar to those described by Fisher, Ingram, Hare and Ranson (1935) and others who have studied degenerative changes in the posterior lobes of cats, dogs and monkeys after clamping the pituitary stalk or destroying the supra-optic nuclei (Mahoney and Sheehan, 1936; Keller, Noble and Hamilton, 1936).

The pars intermedia appeared to be hypertrophied. In comparison with the anterior and posterior lobes it was much larger than normal (fig. 3-E). Other peculiarities were noted. The cells were larger and some of them stained more deeply than normal. They consequently resembled slightly the basophils of the anterior lobe. Most striking was the granular

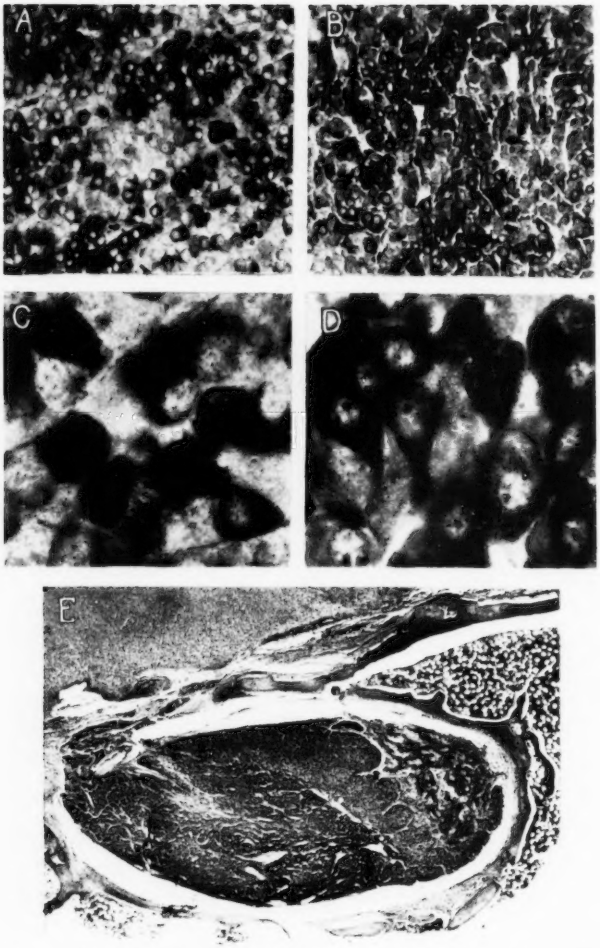


Fig. 3. A. Section (approx. × 320) through anterior pituitary of a normal rabbit. The dark cells are eosinophils.

B. Section through anterior lobe of stalk-cut rabbit (same magnification as A). Long cytoplasmic processes extend out from many of the dark eosinophil cells.

C. Higher magnification (approx. × 1480) of the eosinophils of the normal gland.

D. This figure (magnification, approx. × 1490) shows elongated eosinophils (2) characteristic of the stalk-cut gland. Above the two cells with cytoplasmic processes is an eosinophil of normal shape. It can be seen that the cytoplasm of these cells is less granular and less heavily stained than that of the eosinophils of the normal pituitary.

E. Section of the entire pituitary of a stalk-cut rabbit. Scar tissue can be seen between the remnant of the stalk and the brain. There is a region of scarring in the middle of the anterior lobe. The apparent hypertrophy of the pars intermedia is obvious. The pars posterior is shrunken and abnormal in appearance.

mass which had formed at one pole of the nucleus in many of the cells. This mass of granules was generally about the size of the nucleus or even a little larger. Such granular aggregates were never seen in pars intermedia cells of normal glands. They appeared to be characteristic of the glands of animals with partially or completely severed pituitary stalks.

Anterior lobe changes were studied in greater detail. The eosinophils showed the most marked abnormality. Their granules had almost completely disappeared. The cells retained their peculiar affinity for dyes, however, and could be easily distinguished from the chromophobes. The number and distribution of the eosinophils was within the normal range of variation. In the glands of stalk-cut rabbits long thin cytoplasmic processes extended from many of the eosinophil cells out along the walls of the sinusoids. In many cases these processes were longer than the diameter of the cells. It was found that these changes had begun in glands obtained from animals two days after stalk transection. On the seventh day some eosiniphil cells showed short cytoplasmic processes; a few cells were completely degranulated, others were still practically normal while numerous others showed evidence of partial degranulation. In these the granules were three to four times normal size and had the appearance of globules rather than of discrete granules. By the fourteenth day the maximum state or abnormality had been attained. Figure 3 shows sections of the anterior lobes of normal and stalk-cut rabbits.

Normal basophils were present in all glands but appeared to be diminished in number. Some basophilic cells did show abnormalities; a few were smaller than normal and had pycnotic nuclei while others had the appearance of "castrate" cells with large negative images of the Golgi apparatus. The chromophobes appeared to be normal in structure and number.

The glands from four rabbits which had ovulated as a result of excessive excitation after partial stalk transection were studied carefully. In two of these glands the eosinophils were completely degranulated and showed the characteristic abnormal cytoplasmic processes. In a third, the granules were swollen to three or four times their normal size as if undergoing dissolution. In the fourth preparation normal granulated eosinophils were present. A few cells showed signs of partial degranulation but most of them were completely normal. These abnormalities were similar to those observed in the glands of rabbits with completely severed pituitary stalks.

The cause and significance of these histological changes is not known and it seems useless to speculate concerning them. It suffices to state that in the glands from two of the animals with partially transected stalks the eosinophils were more nearly normal than those of the glands of stalk-cut animals. In two other preparations from rabbits which had also ovulated

after partial section of the stalk the eosinophils were fully as abnormal as those of animals with completely cut stalks. There were a few abnormal basophils in all of these glands but the majority of the anterior lobe basophils were normal in both the stalk-cut animals and those rabbits with only partially transected stalks.

Evidence of functional activity of the anterior pituitary after stalk section. Since a study of the glands of rabbits with transected pituitary stalks showed circulatory changes and histological abnormalities which conceivably could have been caused by damage to the circulation, it was felt that these changes probably indicated functional deficiencies. These deficiencies might explain the inability of these animals to ovulate after mating. Anterior lobe deficiencies, however, should be detectable in a number of ways. A series of physiological and histological tests were therefore carried out in an effort to judge the degree of any functional deficiency which might have been present.

Atrophy of the genital organs in adult rabbits and failure of general body growth and development in young animals regularly follow partial or complete pituitary ablation (Smith, 1932; Sakamoto and Saito, 1932). In the rabbits with severed pituitary stalks, however, body weight and growth were normal. The ovaries, thyroids, adrenals, and genital tracts were generally of normal weight (table 2) and showed no obvious histological abnormalities. Figure 2-C and D show segments from the uteri of a normal and a stalk-cut animal. The endometrium of the latter is within the limits of normal variations. The ovaries of a few of the animals whose pituitary stalks had been cut showed fewer large follicles than are usually seen in normal rabbits. In these animals the genital tracts were moderately atrophic. Many of the rabbits did not show these changes and the tissues most certainly did not resemble those of hypophysectomized animals.

Harris (1937) has recently reported a study of four female and two male rabbits with "lesions of varying extent in the stalk." None of these rabbits mated. Immediately following the operation "the animals were, to outward appearances, normal in every way," but anorexia eventually developed, they became emaciated and death occurred from two to seven months after the operation. On autopsy it was observed that extreme atrophy of the sexual organs had occurred and that the pituitaries showed unmistakable degenerative changes. My results are not in agreement with those of Harris on this point. Section of the pituitary stalk does not necessarily produce, in either male or female rabbits, atrophy of the sexual organs, lack of sexual desire or ability to mate nor does it produce such a great degree of histological abnormality of the pars anterior as that described by Harris.

It is well known that alterations in carbohydrate metabolism result

from partial removal of or injury to the anterior lobe of the hypophysis. Extreme sensitivity to insulin (Cope and Marks, 1935) and a change in sugar tolerance (Greeley, 1935) are characteristic of pituitary insufficiency. In the stalk-cut animals, however, the blood sugar level and sugar tolerance were normal and there was no increased sensitivity to insulin (table 3). The normality of the responses of the animals to these tests precludes any possibility of extensive anterior lobe injury (Houssay, 1936).

TABLE 2

Weights of organs—in grams

CONDITION OF RABBIT	BODY WEIGHT	ENTIRE GENITAL TRACT	RIGHT OVARY	LEFT OVARY	THYROID GLAND	LEFT ADRENAL GLAND	LEFT SUB-MAXILLARY GLAND
	kgm.						
Normal	3.35	10.05	0.260	0.250	0.250	0.260	0.630
	2.60	9.57	0.310	0.330	0.230	0.185	0.860
	2.19	8.32	0.125	0.130	0.210	0.205	0.655
	1.78	4.29	0.120	0.110	0.240	0.350	0.520
	2.15	7.84	0.200	0.170	0.370	0.330	0.545
	2.63	5.03	0.140	0.140	0.260	0.510	0.570
	2.37	9.13	0.170	0.174	0.200	0.305	0.855
	2.66	7.80	0.155	0.140	0.280	0.250	0.620
	2.50	16.00	0.160	0.175	0.220	0.250	0.700
	2.90	9.76	0.175	0.220	0.140	0.120	0.560
Averages	2.51	8.78	0.182	0.184	0.240	0.277	0.652
Pituitary stalk cut	2.08	4.50	0.090	0.110	0.135	0.170	0.490
	2.42	3.19	0.160	0.165	0.260	0.210	0.610
	2.50	10.02	0.230	0.230	0.350	0.185	0.600
	4.02	13.55	0.420	0.390	0.370	0.350	0.800
	1.45	2.77	0.100	0.099	0.130	0.350	0.395
	3.20	15.25	0.175	0.200	0.180	0.330	0.775
	2.20	8.00	0.102	0.115	0.295	0.195	0.695
	1.17	3.20	0.090	0.106	0.170	0.220	0.470
	2.27	4.55	0.120	0.110	0.290	0.350	0.640
	2.47	7.85	0.150	0.120	0.210	0.400	0.575
Averages	2.38	7.29	0.164	0.165	0.239	0.276	0.605

One of the chief indications that the gonadotropic function of the pituitaries of the stalk-cut rabbits remained normal was the ripening of ovarian follicles. This occurred to such a degree that ovulation promptly resulted on injection of pregnancy urine itself or pregnancy urine extract. Ovulation does not occur as a result of such injections in hypophysectomized rabbits in the chronic state (Hinsey and Markee, 1933a). No attempt was made to determine the quantity of material necessary to produce the ovula-

tion, but 0.5 cc. of Antuitrin-S or 5 cc. of pregnancy urine, injected intravenously, did produce full follicular luteinization in rabbits which weighed approximately three kilograms. This proves that after complete stalk section the ovaries are capable of ovulating and that sufficient pituitary activity occurs to maintain the gonads. The animals which were

TABLE 3

Sensitivity to insulin

RABBIT'S NUMBER	STATE OF ANIMAL	BLOOD SUGAR		AMOUNT OF INSULIN	BLOOD SUGAR						
		Time before injection			Time after injection						
		20 minutes	1 minute		5 minutes	30 minutes	60 minutes	120 minutes	180 minutes	240 minutes	350 minutes
		mgm. per cent	*mgm. per cent*	*units per kgm.*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
51	Pituitary stalk cut	87	83	2	70	58	64	52	63	71	87
60	Pituitary stalk cut	80	82	2	77	59	31	29†	49	60	
64	Pituitary stalk cut	85	84	2		51	42	41	37	30†	60
66	Pituitary stalk cut	98	94	2	90	73	70	78	88	133	141
67	Pituitary stalk cut	100	95	2	89	87	76	69	93	91	94
71	Pituitary stalk cut		85	2		61	58	56	42	48	64
108	Pituitary stalk cut		100	2	84	65	49	47		65	
195	Pituitary stalk cut	90	95	2	98	67	63		53		115
196	Pituitary stalk cut		91	2	107	70	54	43		110	
107	Pituitary stalk partly cut		88	2	86	72	57		68		76
188	Pituitary stalk partly cut	90	88	2	87	69	70		73	82	
N-2	Normal	120	110	2	70	63	52	67	80	110	125
N-2	Normal	100	100	2	90	45	55	40	34*†		70
31	Castrate		81	2	78	66	51	36†		52	57
35	Castrate	75	75	2	75	54	53	32*†		66	
52	Castrate	80	83	2	68	58	31†	26*†	57	*†	‡
200	Partially hypophysectomized		84	2	81	74	43	34*†	45	*†	

* Injection of sugar.

† Hypoglycemic symptoms.

‡ Dead.

caused to ovulate by injections of pregnancy urine had, nevertheless, failed to ovulate on repeated matings.

This series of experiments indicates that the pituitary abnormalities produced by stalk transection were not sufficient to create functional deficiencies detectable by these relatively sensitive tests. If a pituitary deficiency due to injury is responsible for the failure of animals to ovulate

then the injury must be either very specific or the mechanism of ovulation must be more easily disturbed than are other reaction processes.

Discussion. Evidence that coitus and the attendant emotional excitement cause an ovulation-producing substance to be discharged from the anterior lobe appears to be conclusive.

The experiments here described prove beyond doubt that the sympathetic innervation of the anterior lobe is not essential to this coitus-induced activation of the gland.

The fact that in this series of experiments cutting the pituitary stalk prevented ovulation suggests that this pathway is essential to the anterior lobe activation. These experiments contribute something to the understanding of the mechanism of excitation but do not settle the matter completely.

Animals with severed pituitary stalks were studied during all the various seasons of the year. Their failure to ovulate could, therefore, not have been due to mating out of the normal breeding season. It is commonly stated that normal rabbits mate and ovulate more readily in the spring and fall than during the summer or winter months. In my rabbit colony little difficulty has been encountered in obtaining mating and ovulation during all seasons of the year. On hot summer days the animals do not mate readily but at night or on cool days mating which is followed by ovulation is easily elicited. During all seasons female rabbits occasionally refuse to mate for brief periods but if they are in good health these "anestrus" periods are infrequent and of short duration. I have seldom found that a mature healthy female mates without ovulating. No examples of spontaneous ovulation have been observed among my rabbits.

Unquestionably there are nerve-like fibers of non-sympathetic origin which terminate in the anterior lobe. These fibers reach the pars anterior from the pars intermedia and the stalk but the individual fibers have never been traced proximally beyond the posterior lobe or the stalk; they become lost there in the large fiber masses. The fact that they are never present after the stalk has been severed is the most conclusive evidence available that they originate in the hypothalamus and pass to the gland by way of the stalk. These fibers were found in all the animals studied which ovulated as a result of coitus. After partial transection of the stalk they were present but in diminished numbers. The greater intensity of stimulation required to induce ovulation in these animals fits in nicely with the supposition that the nerve supply had been reduced but not completely destroyed. These experiments can therefore be considered to support the hypothesis that in the rabbit a nervous stimulation of the pituitary normally occurs on mating and that the nerves involved pass from the hypothalamus down the stalk to the anterior lobe of the pituitary.

Dandy (1913) has stated that the sympathetic fibers which innervate the

anterior lobe are derived from the carotid sympathetic plexus and pass along the arteries to the gland. Numerous nerve branches radiate to the stalk from these vessels but the main trunks enter the gland with the arteries. Transection of the stalk probably severs the stalk sympathetic fibers but one would not expect such an operation completely to destroy the sympathetic innervation of the gland since the arteries are uninjured. The silver techniques used in these studies, however, failed to reveal any anterior lobe fibers in the glands of animals whose pituitary stalks had been severed. Nor were the anterior lobe fibers noticeably fewer in the pituitaries of sympathectomized animals. One possible explanation is that the techniques used did not stain fibers of sympathetic origin. Tello (1911) in his study of pituitary fibers employed a similar silver method and he claimed that sympathetic fibers were not satisfactorily differentiated. The problem of differentiating between possible sympathetic and non-sympathetic anterior lobe fibers is a difficult one. Uncertainty concerning this question, however, does not lessen the weight of the evidence that the sympathetic supply to the pituitary is not essential to its gonadotropic function. One recently published experiment of Harris' (1937) contributes to the evidence that the sympathetic connections are not essential to the ovulation-producing activity of the rabbit's pituitary. He was able to produce ovulation by direct electrical stimulation of the hypothalamus in a rabbit from which the superior cervical ganglia had been extirpated.

It is not admitted by all histologists that these anterior lobe fibers are really nerve fibers. There is more general agreement that there are nerves in the pars posterior and pars intermedia. This difference in the richness of innervation of the various lobes might be due to greater difficulty encountered in staining anterior lobe fibers. As has been suggested by Hinsey and Markee (1933b) it is possible but improbable—at least evidence is lacking—that on coitus excitatory impulses are carried to the pars posterior or pars intermedia and that some humoral mediator produced there is responsible for the anterior lobe activity. A number of workers have failed to produce ovulation in the rabbit by the administration of various neuromimetic drugs. While making the studies described in this paper various concentrations of acetyl-beta-methylcholine chloride (Mecholin), with and without preliminary eserinization, were given without effect. Pituitrin and adrenaline likewise failed to produce ovulation in normal estrus rabbits.

The reason that these experiments cannot be considered absolute proof of the nervous activation of the gland is that stalk section interrupts or injures the hypothalamico-hypophyseal portal system. Conceivably this system could carry some specific chemical excitant from brain to pituitary under normal conditions but could not do so following stalk section. The histological changes which occur in the gland may be due

to circulatory abnormalities or to the absence of nerves. Although injections of the gland vessels showed only slight morphological deficiencies in the stalk-cut animals the physiological effect of these could not be estimated. The tests for gland function and the histological examinations of certain glands and organs, the activities of which are dependent to a large measure upon the anterior lobe, failed to reveal any signs of anterior lobe hypofunction. It was also found that injections of pregnancy urine did cause ovulation. This demonstrated that the pituitary gland possessed some gonadotropic activity and that the ovaries were capable of ovulation and normal luteinization. Nevertheless, it is possible that the glandular deficiencies were sufficient to render the anterior lobe unresponsive to stimuli which normally excite ovulation-producing activity and which might still have been present after stalk section. Two of the animals which ovulated after partial stalk transection showed histological and circulatory abnormalities which appeared to be identical with those seen in the non-ovulating females with completely severed stalks. Although this might be considered to be evidence of an identical functional ability it is quite possible that the techniques used did not permit the observation of minute degrees of difference which could have had a profound functional significance. Solution of this problem depends upon the severance of nervous connections between the brain and the pituitary tissues without the production of these histological and circulatory changes. This is being attempted.

Haterius and Derbyshire (1937) and Harris (1937) have been able to produce ovulation in the rabbit by stimulation of various localized areas of the hypothalamus. This strengthens the evidence that a nervous mechanism is involved in the activation of the anterior lobe gonadotropic function but the fiber pathways have not been fully explored as yet.

SUMMARY

Complete removal of the sympathetic chains fails to abolish or even to alter in any significant way the occurrence of ovulation following coitus in the rabbit.

After severance of the pituitary stalk eighteen rabbits failed to ovulate though they mated frequently.

Fibers resembling nerve fibers were found in the anterior lobes of the normal rabbits studied. These fibers were not numerous. They appeared to enter the gland from the stalk. They were present after removal of the sympathetic ganglia which supply postganglionic fibers to the head but were never found after complete stalk transection.

The pituitaries of the animals with transected pituitary stalks showed certain histological and circulatory abnormalities.

A few rabbits (seven) with partially transected stalks ovulated only

after many matings within a brief period of time. The circulatory and histological changes in the pituitaries of some of these animals were apparently as great as those found after complete stalk transection. A few fibers were present in the pars anterior, pars intermedia and pars posterior. It is quite possible that these fibers mediated the ovulation-producing activation of the anterior lobe following coitus.

It is felt that these experiments support the hypothesis that in the normal rabbit a nervous stimulation of the pituitary occurs on mating and that the nerves involved pass down the stalk from the hypothalamus to the anterior lobe. Proof of this cannot be considered complete until these fibers can be selectively stimulated and ovulation thus produced or until coitus-induced ovulation can be prevented by a selective severance of these fibers which does not injure the circulatory connection of the gland.

I am greatly indebted to certain members of the Department of Anatomy of this University for their suggestions and assistance.

REFERENCES

BELLERBY, C. W. Quart. J. Exper. Physiol. **24:** 123, 1934.
BERKLEY, H. J. The Johns Hopkins Hospital Repts. **4:** 285, 1895.
BODIAN, D. Anat. Rec. **65:** 89, 1936.
BRAMBELL, F. W. R. AND A. S. PARKES. J. Physiol. **74:** 173, 1932.
BROOKS, C. McC. This Journal **120:** 544, 1937.
COPE, O. AND H. P. MARKES. J. Physiol. **83:** 157, 1935.
CROLL, M. J. Physiol. **66:** 316, 1928.
DANDY, W. E. Amer. J. Anat. **15:** 333, 1913.
FEE, A. R. AND A. S. PARKES. J. Physiol. **67:** 383, 1929.
FELDBERG, W. J. Physiol. **61:** 518, 1926.
FISHER, C., W. R. INGRAM, W. K. HARE AND S. W. RANSON. Anat. Rec. **63:** 29, 1935.
FISHER, C., W. R. INGRAM AND S. W. RANSON. Arch. Neurol. and Psychiat. **34:** 124, 1935.
FLETCHER, W. M. J. Physiol. **22:** 259, 1898.
FRIEDGOOD, H. B. AND G. PINCUS. Endocrinol. **19:** 710, 1935.
FRIEDMAN, M. H. This Journal **89:** 438, 1929.
GREELEY, P. O. Proc. Soc. Exper. Biol. and Med. **32:** 1070, 1935.
HAIR, G. W. Anat. Rec. **67:** 21 P., 1937.
HAMMOND, J. Reproduction in the rabbit. Oliver and Boyde, Edinburgh, 1925.
HARRIS, G. W. Proc. Roy. Soc. **122B:** 374, 1937.
HATERIUS, H. O. AND A. J. DERBYSHIRE. Proc. Am. Physiol. Soc. This Journal **119:** 329, 1937.
HINSEY, J. C. AND J. E. MARKEE. This Journal **106:** 48, 1933a. Proc. Exper. Biol. and Med. **31:** 270, 1933b.
HOUSSAY, B. A. New England J. Med. **214:** 971, 1936.
KELLER, A. D., W. NOBLE AND J. W. HAMILTON. This Journal **117:** 467, 1936.
MAHONEY, W. AND D. SHEEHAN. Brain **59:** 61, 1936.
McPHAIL, M. K., A. S. PARKES AND W. E. WHITE. J. Physiol. **79:** 180, 1933.
PENFIELD, W. G. Cytology and cellular pathology of the nervous system. Vol. 2. Paul B. Hoeber Inc., N. Y. 1932.

PINES, J. L. Ztschr. f. d. ges. Neurol. u. Psychiat. **100:** 123, 1926.
RAMON Y CAJAL. Hypophysis. Histologie du Système Nerveux, French ed., Paris **2:** 487, 1894.
RANSON, S. W. Am. J. Anat. **12:** 67, 1911–12.
ROUSSY, G. AND M. MOSINGER. Ann. de Med. **33:** 301, 1933.
SAKAMOTO, A. AND G. SAITO. Ztschr. f. d. ges. Exper. Med. **80:** 601, 1932.
SMITH, P. E. Sex and internal secretions. E. Allen. Chap. XV. Baltimore, 1932.
SMITH, P. E. AND W. E. WHITE. J. A. M. A. **97:** 1861, 1931.
TELLO, F. Trabajos del Lab. de Investigaciones Biologicos Univ. de Madrid. **9–10:** 145, 1911.
WISLOCKI, G. B. AND L. S. KING. Am. J. Anat. **58:** 421, 1936.

RESPONSE TO COLD AFTER REDUCTION OF ADRENAL TISSUE[1]

S. M. HORVATH, F. A. HITCHCOCK AND F. A. HARTMAN

From the Department of Physiology, The Ohio State University, Columbus

Received for publication July 23, 1937

Among the organs which aid the body in meeting the effects of cold—one of the commonest stresses to which man and animals are subjected—the adrenal is of major importance. Although it is well known that the resistance to cold is much reduced after complete destruction of both adrenals (Gautrelet and Thomas, 1909) it has not yet been proved that the destruction of a single adrenal will produce demonstrable effects. Material presented in this paper shows that it does.

METHODS. The purpose of this investigation was to determine the changes in respiratory metabolism which occur in response to cold both before and after single adrenalectomy. Young male albino rats weighing 80 to 110 grams were used as subjects since they are more susceptible to cold than are older animals. An open-circuit apparatus similar to that described by Carpenter (1931) was used for metabolic determinations. All determinations were made between 9:30 a.m. and 3:00 p.m (Horst et al., 1930). The generalized procedure was as follows. The animal, after an 18-hour fast, was confined in a small glass chamber that was submerged in a constant temperature water bath (29° or 4°C.). The animal's movements were automatically recorded and all periods in which activity occurred were discarded. Outdoor air, after first passing through a wet-test meter, was circulated through the chamber by means of a motor-driven rotary blower. An aliquot part of the outgoing air was collected in spirometers over mineral oil and the balance passed through a second wet-test meter. Samples of the air from the spirometers were dried, stored over mercury, and then analyzed (usually the same day) by means of the Carpenter (1933) modification of the Haldane air-analysis apparatus. The air-analysis apparatus was frequently tested by the analysis of outdoor air. The average results of fifteen such analyses were as follows: O_2, 20.939 and CO_2, 0.032 per cent. The standard deviation for O_2 was $\pm$ 0.001 and for CO_2, 0.002, while the maximum deviation was 0.010 for O_2 and 0.003 for CO_2. Three to five 15-minute periods were run and the

[1] Aided by a grant from The Rockefeller Foundation.

results of the two periods which gave the best agreement taken as the basal heat production.

The animals were kept at an environmental temperature of 27–28°C. and their basal heat production measured at 29°C. They were then placed in a cold-room maintained at a temperature of 4°C. for periods ranging from 2 to 72 hours and their metabolism under basal conditions measured again at 29°C. Single adrenalectomy was then performed under ether anesthesia and the above procedure repeated six or eight days later.

A second series of experiments was carried out in which the heat production of rats was determined at 4°C. both before and after single adrenalectomy. Tests on a few animals were run continuously for several hours and the procedure then was as follows: 1, three 15-minute periods were run at 29°C.; 2, the animal was transferred to an environmental temperature of 4°C. and from four to six 30-minute periods run, and 3, the animal was returned to an environmental temperature of 29°C. and two or three 30-minute periods run.

The results were calculated in terms of calories per square meter per 24 hours. The surface area of the rats was calculated by means of the Meeh formula, using 9.1 as the constant (Horst, Mendel and Benedict, 1930).

Colonic temperatures of a group of 12 animals were obtained by means of a small centigrade thermometer which was inserted into the rectum a distance of 30 mm. Data were obtained in this way at environmental temperatures of 29° and 4°C., both before and after single adrenalectomy.

Results and discussion. The period of time during which a single animal was under observation was so short (average of three weeks) that the possible complicating factor of growth was negligible. Control tests run at intervals of three to four weeks on normal animals showed a maximum difference of 1.5 per cent in calories per square meter of body surface. Two out of three animals gave differences of less than 1 per cent.

Since the total respiratory quotients were within the range usually found in rats under basal conditions, they are not included in any of the tables. The average values for normal animals were: Basal 0.70; in the cold, 0.72; following exposure to cold, 0.72. The average values for rats with one adrenal removed were: Basal, 0.69; in the cold, 0.71; following exposure to cold, 0.70.

Response to cold. When normal animals were exposed to a lower environmental temperature for a period ranging between two and seventy-two hours, the metabolism determined at 29°C. immediately after removal from the cold was without exception increased (see table 1). Although there was considerable variation, only two rats showed an increase of less than 10 per cent (9.8 per cent in rat 1 and 7.2 per cent in rat 11).

The length of time during which an animal was exposed to a temperature

of 4°C. seemed to have little influence upon the response, as is shown by a comparison of rats 5, 6, 8 and 13 which had almost identical increases in heat production (39 to 44 per cent) although the time of exposure to cold varied from two to forty-eight hours.

One control animal (rat 20) was subjected to a mock operation. The response to cold was not materially altered by this procedure.

TABLE 1

A comparison of the stimulating effect of exposure of albino rats to cold (4°C.) measured at 29°C.

RAT	DURATION OF EXPOSURE	NORMAL			SINGLE ADRENALECTOMIZED			DECREASE IN RESPONSE
		Heat production in Cals. per square meter per 24 hours						
		Before	After	Per cent	Before	After	Per cent	
	hours							
9	72	940	1200	+28	996	1061	+7	21
8	48	798	1120	+40	746	1040	+39	1
15	48	880	986	+12	873	963	+10	2
6	36	816	1173	+44	790	856	+8	36
						1012	+28	16
7	36	869	1061	+22	893	861	−4	26
2	24	890	997	+12	912	925	+1	11
3	24	929	1036	+12	929	949	+2	10
4	24	855	1140	+33	898	993	+11	22
5	24	796	1123	+41	829	1074	+29	12
10	24	849	945	+11	909	928	+2	9
1	18	889	975	+10	889	951	+7	3
						897	+1	9
11	3	907	973	+7	937	897	−4	11
12	2	829	1039	+25	829	833	+1	24
13	2	883	1226	+39	Died			
14	2	881	1013	+15	900	895	−1	16
					882	873	−1	16
16	2	799	941	+18	805	851	+6	12
Average.....		863	1059	+23	876	937	+7	16
20	72	913	994	+9	929	1006	+8	*

* Mock operation.

A total of 88 basal tests on 16 animals gave an average heat production of 863 Calories per square meter per 24 hours while in 44 tests following exposure to 4°C. the heat production rose to 1059 Calories, an increase of 196 Calories or 22.7 per cent, irrespective of length of exposure. This was a considerably greater effect than the 15 per cent increase in heat production reported by Schwabe and Emery (1937) who, however, used a higher temperature (7–12°C.).

Unilateral adrenalectomy produced no significant change in the basal heat production. The average for the entire group was increased only about 1 per cent, a figure well within the experimental error.

After determinations of basal rate these singly adrenalectomized animals were exposed to an environmental temperature of 4°C. for periods of the same duration as in control tests and their heat production then measured at 29°C.

Three of the sixteen animals (7, 11, 14) failed to show any stimulation, the heat production actually being slightly lower after exposure to cold. It should be noted that rat 11 showed the least increase when the stimulating effect was measured previous to the removal of one adrenal.

In the remaining 13 animals the increased caloric production varied from 0.5 to 39 per cent. Here, as in intact rats, these values bear no relation to the duration of the exposure. The average increase in heat production in single adrenalectomized animals following exposure to cold was 8.3 per cent.

Rats 1, 6 and 14 were studied on two occasions following the removal of one adrenal. In rats 1 and 14 the heat production after the second exposure was somewhat lower than that resulting from the first exposure. However, in rat 6 following the second exposure, a larger percentile increase was obtained (table 1).

A comparison of the stimulating effects of low temperatures on the heat production of normal and single adrenalectomized rats showed that in every case animals with the smaller amount of adrenal tissue gave less response than did normals.

These experiments demonstrate that removal of one adrenal decreases the ability to produce heat in response to cold. The average difference between the stimulating effect of cold in normal and singly adrenalectomized animals was 15.8 per cent.

Immediate effect of cold. When normal rats were exposed to a temperature of 4°C. they responded by a greatly increased heat production. This effect seemed to be independent of shivering. The caloric output varied from 2100 to 2900 Calories per square meter per 24 hours. The previous basal determinations for these animals averaged 894 Calories per square meter per 24 hours. The mean increase was 170 per cent, the range being from 245 to 115 per cent (see table 2).

Rat 7A, after a mock operation, was studied on two different occasions. In both instances there was a greater per-cent increase in heat production while in the cold than was observed before the operation. On the first test the heat production was 22 per cent higher than before the operation, while in the second test the increase was 7 per cent. It will be recalled that a similar type of response was observed in rat 20. This would indicate that the operation *per se* tends to increase the response.

When rats were unilaterally adrenalectomized and their heat production measured at 4°C., there was a noticeable decrease in the number of calories produced. The caloric output varied from 1970 Calories to 2480 Calories per square meter per 24 hours. The mean increase was 139 per cent (range 202 to 110 per cent). Rat 8A was the only animal in this series to show an increase in the magnitude of the response to cold following the removal of one adrenal. The loss of one adrenal resulted in an average decrease of 31 per cent in the response to cold (table 2).

Experiments carried out on three animals, both before and after unilateral adrenalectomy, in which consecutive thirty-minute periods were run for several hours immediately after the animals were transferred to an

TABLE 2

Comparison of heat production in normal and singly adrenalectomized albino rats at 29° and 4°C.

RAT	DURATION OF EXPOSURE TO COLD	NORMAL			SINGLY ADRENALECTOMIZED			CHANGE IN RESPONSE
		Heat production in Cals. per square meter per 24 hours						
		At 29°C.	At 4°C.	Per cent change	At 29°C.	At 4°C.	Per cent change	
1A	18	924	2814	205	977	2439	150	−55
10	18	849	2268	167	909	1995	119	−48
4A	3	883	2513	185	889	2164	144	−41
11	3	977	2099	115	937	1969	110	−5
5A	2	878	2391	172	894	2123	138	−34
6A	2	847	2919	245	821	2479	202	−43
8A	2	916	2123	132	967	2331	141	+9
14	3	881	2168	146	900	1995	122	−24
					863	1976	129	−17
Average		894	2412	170	906	2163	139	−31
7A*	2	829	2221	168	862	2476	187	+19
					867	2357	172	+4

* Mock operation.

environmental temperature of 4°C., indicate that not only do unilaterally adrenalectomized animals show a lowered maximum heat production in the cold, but this maximum is attained more slowly than in normal animals. Control animals reached their maximum heat production after exposure to the cold for one hour or less, while with single adrenalectomized animals an exposure of at least twice this duration was necessary before the maximum level of heat production was reached.

Body temperature. Adrenalectomized animals are unable to maintain normal body temperature on prolonged exposure to cold (Wyman and

tum Suden, 1929). The literature contains no data on unilaterally adrenalectomized animals. A study made on 12 animals before and after single adrenalectomy showed no significant difference in their ability to maintain normal body temperature at either 29° or 4°C. The average body temperature for operated animals was 0.1°C. lower than for their controls at both temperatures.

Conclusions. Our observations demonstrate that the presence of both adrenals in the organism makes it possible to meet the stress of cold more adequately than does one, only. One adrenal suffices for the quiet life of the ordinary laboratory rat but two of them are a distinct advantage and may be necessary in certain prolonged stresses.

Animals do not always show a fall in metabolism following reduction of adrenal tissue. Marine and Bauman (1921, 1922) found in rabbits that partial destruction of the adrenal cortex was followed by a 23 per cent increase in metabolism. This may be a species difference because Barlow (1924) found no change or a slight drop in the basal oxygen consumption of singly adrenalectomized cats. Brownell (personal communication) finds in dogs, as we have found in rats, that there is no change in basal rates after unilateral adrenalectomy. The difference in caloric response of normal and singly adrenalectomized rats to cold might be attributed to a lack of epinephrin. However, it is more likely due to an inadequate secretion of cortin because administration of epinephrin in doubly adrenalectomized rats has little effect on the resistance to cold (Hartman, Brownell and Crosby, 1931), while injected cortin enables adrenalectomized rats to produce heat almost as well as normals. Wyman and tum Suden (1929) were also able to demonstrate that adrenalectomized rats could be protected against short exposure to cold by autoplastic cortical transplants.

The ability of unilaterally adrenalectomized rats to maintain normal body temperature in the cold despite a lowered heat production seems to point to good physical regulation of body temperature. The fluffing of the hair and constriction of the cutaneous blood vessels probably act to prevent excessive heat loss.

The present work indicates that a rat requires all of its adrenal tissue in order to produce the additional heat required on prolonged exposure to cold.

SUMMARY

Studies of the respiratory metabolism of normal and unilaterally adrenalectomized albino rats at environmental temperatures of 29° and 4°C., and at 29° after exposure to 4°C. for periods of from two to seventy-two hours show that:

1. The basal heat production of albino rats is unchanged following the removal of one adrenal.

2. Normal albino rats after exposure to an environmental temperature of 4°C. have an average increase in heat production measured at 29°C. of about 22 per cent. The corresponding increase in heat production of unilaterally adrenalectomized animals is only about 7 per cent.

3. At an environmental temperature of 4°C. the maximal heat production of unilateral adrenalectomized rats was attained more slowly and was lower than that of normal animals.

4. The colonic temperatures of normal and singly adrenalectomized rats remains practically unchanged at environmental temperatures of both 4° and 29°C.

REFERENCES

BARLOW, O. W. This Journal **70:** 453, 1924.
BENEDICT, F. G. AND G. MACLEOD. J. Nutrition **1:** 367, 1929.
CARPENTER, T. M. Abderhalden's Handbuch d. Biol. Arbeitsmethoden Abt. IV, 593, 1933.
CARPENTER, T. M. AND E. L. FOX. Arbeitphysiol. **4:** 527, 1931.
GAUTRELET, J. AND L. THOMAS. Compt. rend. Soc. de Biol. **67:** 386, 1909.
HARTMAN, F. A., K. A. BROWNELL AND A. A. CROSBY. This Journal **98:** 674, 1931.
HORST, K., L. B. MENDEL AND F. G. BENEDICT. J. Nutrition **3:** 177, 1930; Ibid. **7:** 277, 1933.
HORVATH, S. M. Proc., This Journal **119:** 339, 1937.
MARINE, D. AND E. J. BAUMAN. This Journal **57:** 135, 1921; **59:** 353, 1922.
RING, G. C. Proc., This Journal **116:** 129, 1936.
SCHWABE, E. K. AND F. E. EMERY. Anat. Record **67:** Supp. 3, 46, 1937.
WYMAN, L. C. AND C. TUM SUDEN. This Journal **89:** 363, 1929.

INCREASED SODIUM CHLORIDE APPETITE IN PREGNANT RATS

BRUNO BARELARE, JR. AND CURT P. RICHTER

From the Psychobiological Laboratory, Henry Phipps Psychiatric Clinic, Johns Hopkins Hospital, Baltimore, Md.

Received for publication July 23, 1937

It has been shown that rats in various altered physiological states show an increased appetite for substances which make for the well-being of the animal. The increased appetite which adrenalectomized rats display for sodium chloride (Richter, 1936), and the increased appetite which parathyroidectomized rats display for calcium (Richter and Eckert, 1937), are instances of this.

With this in mind, a study of the animal's appetite during the period of gestation was undertaken. Heretofore, the studies of metabolism during pregnancy have been based on chemical analyses of blood, bones and excreta. By means of the appetite method it is possible to obtain a picture of the various needs of the animal in a much simpler manner.

The following studies were made on the appetites of pregnant animals given free access to various substances.

METHODS. Twelve female rats were placed in cages containing 3 receptacles for solid foods and inverted graduated bottles for solutions. The following substances were offered in the separate compartments: casein (protein), sugar (carbohydrate), olive oil (fat), dry yeast (vitamin B), cod liver oil (vitamins A and D), calcium lactate solution (2.4 per cent), sodium chloride solution (3 per cent), and water. The animals were given free access to these substances and allowed to make their own choice. In previous experiments it had been found that rats were capable of adjusting themselves very well to such a dietary regime, as evidenced by good growth, normal sex cycles and spontaneous activity (Richter, Holt and Barelare, 1937). The intake of the various materials was measured daily and the animals were weighed weekly. Vaginal smears were studied every day. After a period of time which was allowed for the rats to become accustomed to the cages, the animals were mated. The matings were conducted as follows: the female experimental rat was removed to a regular breeding cage on the night in which she went into heat. The cage contained a water bottle but no food whatsoever. An active male was put in and allowed to remain with her until morning, at which time she was returned to her activity cage.

Five of the animals were allowed to become pregnant a second time and similar studies were made during the second pregnancy.

RESULTS. A definite and very constant increase in sodium chloride intake was noted. The results for the individual animals are shown in the accompanying table (table 1). It will be noted that on the average the animals took over twice as much sodium chloride during the first ten days of gestation as they did during the ten day period immediately preceding

TABLE 1

Average daily sodium chloride intake

RAT NUMBER	DAYS AFTER CONCEPTION ON WHICH SALT RISE STARTED	FOR 10 DAYS BEFORE CONCEPTION		FOR FIRST 10 DAYS OF GESTATION		FOR SECOND 10 DAYS OF GESTATION		FOR FIRST 10 DAYS POSTPARTUM	
		In cubic centimeters of 3 per cent solution	In milligrams	In cubic centimeters of 3 per cent solution	In milligrams	In cubic centimeters of 3 per cent solution	In milligrams	In cubic centimeters of 3 per cent solution	In milligrams
1	3	1.7	51	3.4	102	8.6	258	4.7	141
2	8	7.0	210	12.3	369	10.0	300	7.4	222
3	4	2.0	60	6.0	180	8.0	240	1.9	57
4	3	0.6	18	2.8	84	5.3	159	2.0	60
5	2	1.2	36	6.4	192	6.9	207	3.8	114
6	2	2.9	87	6.5	195	7.9	237	3.9	117
7	3	1.4	42	4.8	144	8.6	258	2.7	81
	2	0.8	24	5.3	159	9.8	294	3.8	114
8	12	3.9	117	4.4	132	4.9	147	1.5	45
	10	0.3	9	1.6	48	3.7	111	3.0	90
9	2	1.4	42	4.4	132	9.5	285	4.3	129
10	12	8.3	249	7.8	234	11.6	348	3.7	111
	2	2.8	84	6.7	201	9.3	279	6.0	180
11	10	0.7	21	1.9	57	4.4	132	1.6	48
	12	1.6	48	2.4	72	4.3	129	2.9	87
12	2	1.3	39	4.2	126	5.2	156	2.1	63
	4	0.7	21	1.9	57	5.3	159	4.8	144
Average		2.3	69	4.9	147	7.3	219	3.5	105

the fertilization. During the second half of the gestation, they took over three times as much. In the ten days immediately following the casting of the litter, the intake decreased, reaching an average level only slightly above the pregestational level. A typical record of one animal, showing the daily intake of sodium chloride solution before, during and after pregnancy, is included (fig. 1).

In 65 per cent of the pregnancies (11 out of 17) the increase in the sodium chloride intake began about the third day after fertilization. In the re-

maining 35 per cent the increase did not start until the tenth to the twelfth day.

Discussion. The significance of this increased sodium chloride appetite during pregnancy is not clear. The fact that it occurs so consistently and is purely voluntary on the part of the animal might reflect a definite need for salt in pregnant animals. It is well known that the term "acidosis of pregnancy" does not mean an accumulation of excess acids in the blood; rather, it is a relative acidosis, depending on the decreased base content. It has been reported that during pregnancy there is a diminution in both

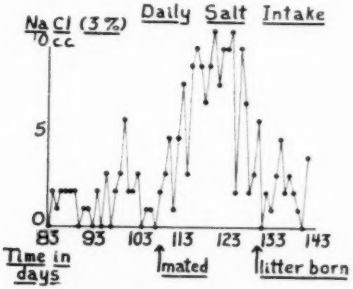


Fig. 1. Typical record of an animal showing the daily intake of 3 per cent sodium chloride solution during the period of gestation.

anion and cation content of the blood. Among the cations, the sodium content suffers the greatest reduction, whereas among the anions the serum bicarbonate, serum protein, and organic acid are reduced (Oard and Peters, 1929). It appears highly probable that the animal in some way reacts to this reduction in electrolytes by voluntarily ingesting more sodium chloride. There is no reason, of course, for suspecting that the sodium chloride increase during pregnancy is the only change in appetite. In fact, some evidence for an increase in calcium intake has already been observed in the course of the above experimentation. Further experiments are now being conducted with regard to this point.

SUMMARY

1. The sodium chloride appetite of twelve female rats during pregnancy was studied by the method of self-selection.

2. On the average, animals ingested over twice as much sodium chloride solution (3 per cent) in the ten day period after conception as in the ten day period before conception, and ingested over three times as much in the second half of the period of gestation. The sodium chloride intake in the first ten days postpartum fell back to a level only slightly above the pregestational level.

3. The probable rôle played by this increased sodium chloride intake is discussed with regard to the decreased electrolyte content of the blood sera of pregnant females.

4. A suggestion that other changes in appetite occur during pregnancy is made with special reference to already observed changes in the appetite for calcium.

REFERENCES

RICHTER, C. P. Am. Jour. Physiol. **115**: 155, 1936.

RICHTER, C. P. AND J. F. ECKERT. Endocrinology **21**: 50, 1937.

RICHTER, C. P., L. E. HOLT, JR. AND B. BARELARE, JR. Proc. Am. Physiol. Soc., April, 1937, p. 132.

OARD, H. C. AND J. P. PETERS. J. Biol. Chem. **81**: 9, 1929.

THE EFFECT OF INTRAPERITONEAL INJECTIONS OF RINGER'S SOLUTION ON KIDNEY WEIGHT IN RATS[1]

PAUL L. STIER AND J. M. HAYMAN, JR.

From the H. K. Cushing Laboratory of Experimental Medicine in the Department of Medicine of Western Reserve University and the Medical Service of Lakeside Hospital, Cleveland, Ohio

Received for publication July 24, 1937

In an extensive investigation of the factors which determine renal weight in the rat, MacKay and his associates showed that the rate of growth of the kidney can be accelerated or inhibited by factors which have little effect on growth in general. One of the most striking of these is the protein intake (1). The excretion of the larger amounts of nitrogen on a high protein diet was at most only a contributory factor, for rats fed an equivalent amount of nitrogen as urea did not exhibit the same degree of renal enlargement (2). Studies of the mechanism of the increased kidney weight with increased protein intake led MacKay and Cockrill (3) to suggest that kidney weight is dependent on "maintenance metabolism." Wilson (4) considered it likely that the hypertrophy was associated with some stage in the intermediary metabolism of protein, probably deamination by the kidney. Feeding thyroid (5) or phosphate (6) also led to an increased renal weight, while NaCl, $CaCl_2$, and $NaHCO_3$ had no effect (6).

Rats on a high protein diet ingest much water and excrete a large volume of urine. The question arises whether the excretion of these large volumes of urine over relatively long periods might be a factor in the increased renal weight in rats fed high protein diets.

White male rats, litter mates, when 100 days old were assigned to one of four groups. One was fed MacKay's "experimental male food" (7) containing 18 per cent protein, chiefly casein; another his highest protein diet, containing 67 per cent protein (1). The other two groups were given the low protein diet, plus daily intraperitoneal injections of Ringer's solution. One group received 10 cc. daily for 15 days and then 20 cc. daily for an equal time, the other group 20 and 40 cc. daily for 15 day periods. After 30 days, the rats were killed, measured, weighed, and heart and kidney weights obtained according to the technique described by MacKay (7).

[1] A preliminary report of some of these experiments was presented before the twenty-eighth annual meeting of the American Society for Clinical Investigation in Atlantic City, New Jersey, on May 4, 1936.

Approximately one-half of each kidney was dried to constant weight at 100°C. and sections cut from the remainders. Each animal was kept in a separate cage, and the food and water consumed determined every other day. Urine was collected from 6 rats in the high Ringer's group and from 5 in each of the others for from 15 to 30 days. The total collections represented 535 rat days.

The low protein group showed the greatest gain in weight during the experiment, animals on the high protein diet the least, with the animals receiving Ringer's solution falling between. The data of the experiment are presented in table 1.

It is obvious that the rats which were given intraperitoneal injections of Ringer's solution and which excreted large volumes of urine did not have any larger kidneys per unit surface area than did the controls. The animals on the high protein diet, on the other hand, had kidneys which were significantly heavier than the controls. Since although the volume

TABLE 1

GROUP	NUMBER OF RATS	CORRECTED BODY WEIGHT		SURFACE AREA		HEART WEIGHT PER SQ. DM.		AVERAGE KIDNEY WEIGHT PER SQ. DM.		WATER RAT PER DAY	URINE RAT PER DAY
		Mean	σ_m	Mean	σ_m	Mean	σ_m	Mean	σ_m		
		gm.	*gm.*	*sq. cm.*	*sq. cm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*cc.*	*cc.*
Low protein	18	239	±9.7	436	±11.7	173	±2.6	170	±2.6	16.6	4.1
High protein	19	207	±10.3	396	±12.9	172	±3.1	221	±6.6	27.9	11.6
Low Ringer	18	220	±10.0	412	±13.2	169	±3.4	171	±5.6	10.6	16.3
High Ringer	18	214	±9.4	405	±11.7	178	±1.1	174	±5.4	8.4	27.7

of urine excreted by these animals was 2.8 that of the controls, it was still below that of the Ringer's groups, the excretion of large volumes of urine is not a factor in the increased renal weight obtained on high protein diets.

Comparison of the kidney weights with those expected from the protein intake on the basis of MacKay's (1) formula showed good agreement. Calculated weight: observed weight, in milligrams of kidney per square decimeter of body surface, in the low protein group was 175:170; high protein group 242:221; low Ringer group 174:171; and high Ringer group 173:174. There was no difference in the percentage of water in the kidneys of the four groups, the means being low protein 72.7; high protein 73.2; low Ringer 73.4; high Ringer 72.6. None of the sections showed any pathological change.

In another group of animals the effect of large urine volumes on the degree of compensatory hypertrophy after unilateral nephrectomy was observed. These animals were from 90–118 days of age at operation, and were killed after 40–43 days. One group was given the diet containing

18 per cent protein, one the 67 per cent protein diet, and the third the low protein diet plus intraperitoneal injections of Ringer's solution twice daily. Animals in this group were weighed twice daily; if this showed undue retention of fluid an injection was omitted or reduced. Consequently the amount of Ringer's solution was not constant, but varied from 20 to 60 cc., usually 40 to 60 cc. daily. While the rats were unfortunately not all of exactly the same age, the different ages were distributed as equally as possible among the groups. The right kidney was removed in each animal under ether anesthesia. While the right kidney is usually

TABLE 2

GROUP	NUMBER OF RATS	MEAN INITIAL				WEIGHT KIDNEY REMOVED PER SQ. DM.		MEAN FINAL				KIDNEY WEIGHT PER SQ. DM.		URINE* PER RAT PER DAY
		Weight		Surface area				Weight		Surface area				
		Mean	σ_m	Mean	σ_m	Mean	σ_m	Mean	σ_m	Mean	σ_m	Mean	σ_m	
		gm.	*gm.*	*sq.cm.*	*sq.cm.*	*mgm.*	*mgm.*	*gm.*	*gm.*	*sq.cm.*	*sq.cm.*	*mgm.*	*mgm.*	*cc.*
Low protein	12	145	±7.8	312	±10.5	204	±5.2	197	±10.3	383	±13.5	253	±8.6	6.0
High protein	15	156	±9.2	330	±13.1	202	±7.7	202	±9.0	394	±12.1	366	±15.8	24.0
Ringer	12	160	±7.3	340	±9.9	203	±4.3	196	±10.4	382	±12.4	298	±13.7	39.6

* For last ten days, mean for 5 rats in each group.

TABLE 3

GROUP	MEAN INTAKE, GRAMS PER SQ. DM. BODY SURFACE PER DAY		MILLIGRAMS KIDNEY WEIGHT PER SQ. DM. BODY SURFACE			DEGREE OF COMPENSATORY RENAL HYPERTROPHY	
	Food	Protein	Calculated due to protein	Observed	Corrected for protein intake	Observed	Corrected for protein intake
						per cent	
Low protein	2.2	0.40	44	253	209	24	38
High protein	2.7	1.81	200	366	176	78	17
Ringer	2.4	0.43	46	298	252	47	65

slightly larger than the left, in our experiments, this difference was not considered in the calculations, the two kidneys being assumed to be of equal weight. The data are given in table 2.

In calculating the surface area at the beginning of the experiment, 5 per cent was subtracted from the observed weight (8). Although the mean weight and surface area of the Ringer group is greater than the others at the beginning of the experiment, the difference is not statistically significant. It is apparent that the increased urine volume produced by Ringer's solution has not been the significant factor in the degree of hypertrophy of rats on a high protein diet after unilateral nephrectomy.

But there is a suggestion in the data that the injections of Ringer's solution may be associated with a greater degree of hypertrophy in the remaining kidney than that in control animals on the same diet. The difference in the mean weight per 100 sq. cm. of surface area in the low protein and Ringer's groups is 45 mgm. and the probable error of this difference 10.9 mgm.

In calculating the degree of compensatory renal hypertrophy after unilateral nephrectomy MacKay, MacKay and Addis (9) corrected the observed weights for the protein intake. A similar calculation on the animals in this experiment is shown in table 3. If this method of calculation be legitimate, it indicates, from the data at hand, that after unilateral nephrectomy injections of Ringer's solution lead to a greater hypertrophy than that found in control animals fed the same diet. Whether this is due to the excretion of the larger amounts of water, or to the salts injected, was not determined. Attempts to substitute glucose for Ringer's solution were successful.

CONCLUSIONS

1. Diuresis provoked by daily intraperitoneal injections of Ringer's solution in rats does not lead to an increase in kidney weight. The increased urine volume on high protein diets is apparently not a factor in the increased renal weight observed on such diets.

2. After unilateral nephrectomy, the degree of compensatory hypertrophy corrected for protein intake, is apparently greater in animals receiving injections of Ringer's solution than in controls.

REFERENCES

(1) MacKay, E. M., L. L. MacKay and T. Addis. This Journal **86:** 459, 1928.
(2) MacKay, L. L., E. M. MacKay and T. Addis. Proc. Soc. Exper. Biol. and Med. **24:** 336, 1927.
(3) MacKay, E. M. and J. R. Cockrill. J. Nutrition **4:** 25, 1931.
(4) Wilson, H E. C. Biochem. J. **27:** 1348, 1933.
(5) MacKay, E. M. and L. L. MacKay. J. Nutrition **4:** 33, 1931.
(6) MacKay, L. L., E. M. MacKay and T. Addis. Proc. Soc. Exper. Biol. and Med. **24:** 130, 1926.
(7) MacKay, L. L. and E. M. MacKay. This Journal **83:** 179, 1927.
(8) MacKay, E. M. and L. L. MacKay. J. Nutrition **3:** 375, 1931,
(9) MacKay, E. M., L. L. MacKay and T. Addis. J. Exper. Med. **56:** 255, 1932.

RECRUITMENT OF NERVE FIBERS

HERBERT S. GASSER

From the Laboratories of The Rockefeller Institute for Medical Research, New York

Received for publication July 26, 1937

Recruitment is a term that has been employed to designate the successive induction of neurons into the service of a reflex (Liddell and Sherrington). It has heretofore not been used in connection with nerve fibers, as no event has been known to which it would apply. However, from time to time the observation has been made—first in some experiments carried out with Dr. J. Hughes in 1933, and recently in experiments with Dr. H. Grundfest—that, when stimulation is started with a train of shocks at a strength near threshold for the nerve as a whole, there is obtained, instead of a series of spikes approximately alike or varying in a rhythmic manner, a series in which the components become successively larger. This augmentation can mean only the progressive addition of new fibers to those already responding; but the mechanism by which the addition takes place has not been clear, as it occurs at shock intervals much longer than the interval during which latent addition is supposed to take place.

Nerve fibers, at least in isolated preparations, are known to fluctuate in excitability, and the possibility was therefore at hand that the shocks were merely picking up the fibers on the upswing of their excitability and holding them after they had once responded, owing to the fact that succeeding shocks were falling in the supernormal phases of the preceding responses. Fibers would thus be added until there was left none, the threshold of which could fall at any time to the level at which the excitation produced by the shocks would be adequate. If this explanation held, the phenomenon would have no great significance. On the other hand, if the phenomenon meant that excitation could actually be summed over long intervals, a fact of considerable importance was disclosed.

Several considerations speak against the adequacy of a theory based on the fluctuation of thresholds. Nerves vary greatly with respect to the readiness with which recruitment takes place, but the nerves displaying it to the highest degree are not those showing the greatest fluctuation of the thresholds of their fibers. (Compare A 4 and B 4 in fig. 4.) Also, the fluctuation of thresholds would not explain delayed entrance of responses into a train of stimuli. If a series of shocks is set at a strength properly below threshold, it is often found that the first response appears only after the fibers have been subjected to a number of stimuli (fig. 1).

Even in the most irritable fibers, excitation must be built up before an impulse is discharged; and it is possible to see in these fibers the progressive summation which over longer periods successively brings in more fibers as the train of shocks is continued.

That a response may first occur after a series of stimuli has been shown beyond peradventure by Blair and Erlanger in single fiber preparations of frog nerve. When a nodal block is effected by anodal polarization, calcium poisoning, or fortuitous deterioration, a number of impulses must come up to the block in succession before one can pass. Erlanger and Blair had previously shown that after the application of a subthreshold induction shock, the threshold for an ensuing shock is lowered for only a millisecond or less, then raised for the next 3 to 4 milliseconds. In contrast, their new summation occurred when the intervals were as long as 100 msec. Thus there was no obvious association of the summation with the subthreshold excitability curve in its known form.

Fig. 1

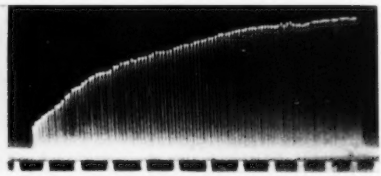


Fig. 2

Fig. 1. Delayed entrance of responses in a tetanus. The train of stimuli starts at subthreshold strength and the first response appears after the tenth shock. More fibers are added by the latter shocks. Veratrinized frog nerve.

Fig. 2. Recruitment of fibers in a veratrinized frog nerve during a tetanus at 47 per second. The recruitment continued for over 6 seconds. Time 0.2 sec.

One reason for the small amount of attention paid to recruitment—or what amounts to the same thing, the summation of multiple stimuli—is the uncertainty of its appearance. In the present investigation, the conditions in which it regularly occurs have been found and the mode of its development has been ascertained.

Methods. Isolated nerves were mounted in a moist chamber and tested with trains of shocks from a thyratron oscillator. The length of the train and the strength and frequency of the shocks could readily be adjusted. The responses were recorded with a cathode ray oscillograph, with the sweep of which the train of shocks was synchronized.

Results. *Frog nerve.* A few experiments only were carried out on frog nerve. Nerves recently removed from the animal and freshly mounted ordinarily did not show recruitment, but after a period of experimentation the phenomenon appeared in a striking manner. It also appeared to a slight degree if the nerves were allowed to soak in Ringer's solution for

some time before mounting. According to previous experience, the most obvious difference between the state of the nerves showing recruitment and the state of those not showing the phenomenon, would be that the former nerves as compared with the latter would have larger negative after-potentials in the course of single responses. With this consideration as a clue, veratrine was tried and, as predicted, it was found to create very effectively the condition necessary for the occurrence of recruitment (fig. 2).

One of the conditions mentioned by Blair and Erlanger as being effective in producing summation was high calcium. Calcium, it will be noted, is one of the substances producing a large negative after-potential (H. T. Graham, 1933). Accordingly its effect on recruitment was ascertained. Two methods were used for preparing the nerves. In one method, calcium chloride was injected into the dorsal lymph sac of the frog and the nerve

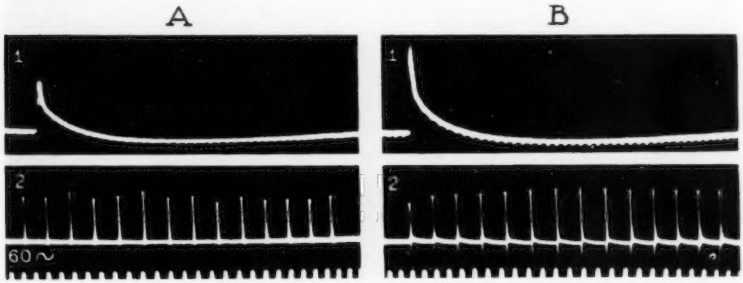


Fig. 3. Recruitment in a calcium-treated nerve. A, control nerve: *1*, after-potential; *2*, responses to a train of shocks slightly above threshold for the nerve as a whole. B, calcium-treated nerve: *1*, after-potential (the corresponding spike height is 55 per cent of that for A *1*); *2*, tetanus showing some recruitment. The initial threshold is much higher than for A *2*.

dissected out after sufficient time had been allowed following the injection for the frog to become limp. In the other method the nerves were immersed for an hour in Ringer's solution containing nine times the normal amount of calcium, as recommended by H. T. Graham, before mounting them in the moist chamber. The result was the same by either method: recruitment appeared. Figure 3 shows the responses of paired nerves. Nerve A was left for an hour in normal Ringer's solution, and nerve B in the calcium-rich solution. A relatively small increase in the negative after-potential was produced in the latter nerve; nevertheless, recruitment, absent in the control, was clearly present in the calcium-treated preparation.

Mammalian nerve. These nerves were kept at 37°C. and in 5 per cent CO_2 in O_2 in order to maintain the pH and the characteristics associated

with pH in a normal state (Lehmann). Like frog nerves, normal and rested mammalian nerves did not permit the production of recruitment, while veratrine brought it out effectively. The routine means for producing the phenomenon, however, was a period of activity. First the responses were observed at varying frequencies of stimulation when the nerve was in the rested state (fig. 4 B). Then the nerve was tetanized through a second pair of electrodes placed on the side of the testing electrodes opposite from the leads. During the period of subnormality

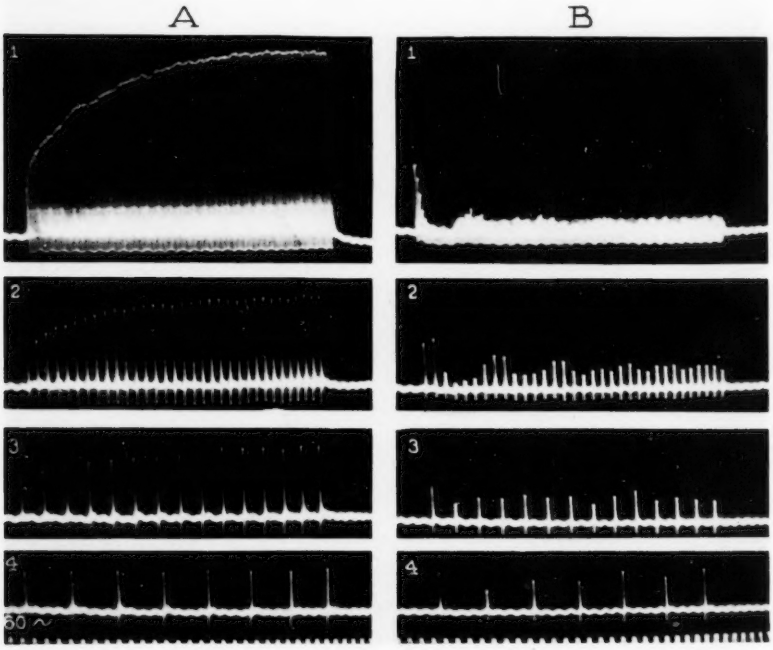


Fig. 4. A, recruitment in a period following intense activity. B, absence of recruitment after the nerve had been rested. The figure shows the effect of frequency of stimulation: *1*, 200 per sec.; *2*, 70; *3*, 30; *4*, 15.

following the tetanus the responses were tested at the same frequencies of stimulation as before. As would be expected, it was necessary to employ stronger shocks in the stimulating trains than were used in the control series. No recruitment was possible in the rested nerves. At the low frequencies random fluctuation of the thresholds was evident, and at the higher frequencies, owing to an insufficient rate of recovery, the fibers were not able to follow the stimuli. On the other hand, after the tetanus, recruitment was present at all frequencies (fig. 4 A).

Recruitment was at its best soon after the termination of the conditioning tetanus, some improvement often taking place during the first minute. Then the recruitment declined again, and the behavior characteristic of resting nerve was finally restored. The cycle was completely reversible, and the transition between the two states could be brought about repeatedly.

In connection with the after-effects of the tetanus there should be mentioned a point which has been known for some time, but which has not previously been described. As shown by Gasser and Grundfest, a tetanus of any length is followed by a positive after-potential which lasts on a declining scale for five minutes or longer. If during this period a single response is set up, the action potential rises from the existing positive level (which is sufficiently steady during the course of a single action); and it is then seen to have a much higher and longer negative after-potential

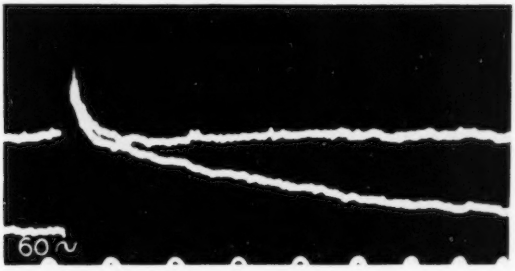


Fig. 5. Comparison of the negative after-potential in a single response in a rested nerve with one in a response following a one minute tetanus. Upper curve, rested nerve. Lower curve, after a tetanus. The difference between the initial levels measures the amount of positive after-potential resulting from the tetanus.

than it has in the rested state of the nerve. The difference is shown in figure 5, made with a D.C. amplifier. The upper figure is the normal after-potential at pH 7.4. Without at any time adjusting the controls, the nerve was tetanized; and after the tetanus a second response was recorded on the film showing the normal response. Thus the two potentials are shown in proper relationship with respect to their background potentials. Later the baseline rose again; and as it did so, the after-potential returned to its normal form.

Other conditions yielding large negative after-potentials on single responses are produced by rapid restoration of a nerve from pH 8.0 to pH 7.4 through the restoration of CO_2 to an $O_2 - CO_2$ mixture from which the CO_2 had previously been withdrawn (Lehmann), and by the restoration of oxygen to an asphyxiated nerve. In both of these conditions recruitment occurs, most obviously, however, in the latter. Figure 6 shows the negative after-potentials before and after an asphyxiation to

the point of complete inexcitability and the responses to trains of stimuli of the same frequency, as the responses appeared in the two states which yielded the after-potentials.

A glance at figure 4 A is sufficient to show that both the rate of recruitment and the total number of fibers recruited are a function of the frequency of stimulation. In this experiment summation was no longer possible at intervals of 60 msec. In other experiments it still occurred at this interval, and in veratrinized nerve it was effective for intervals well over 0.5 sec.

The records in figure 4 illustrate for nerve characteristics which hold for latent addition in general. The laws of summation have been worked out on slow tissues by Lapicque, Chauchard and Chauchard, and Monnier,

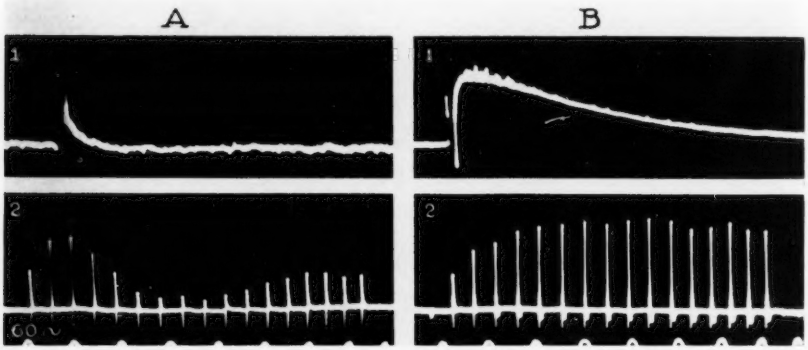


Fig. 6. Recruitment during early recovery from asphyxia. A, normal nerve: *1*, after-potential; *2*, tetanus with shocks slightly above threshold. B, after asphyxia.

and for the myoneural junction by Bremer and Homès. Summation is known to depend upon the strength, frequency, and number of the stimuli, and in addition upon the duration and shape (Monnier) of the stimulating shocks.

The fact that summation is more effective at high than at low frequencies indicates that the process making for summation is more intense at the start and decrements gradually, reaching a value no longer detectable after various intervals up to 100 msec. or longer. This is exactly the characteristic of classical latent addition, only the latter ends after 0.2 to 0.4 msec. (the exact value for mammalian nerve is not at present known) and is succeeded by a period of postcathodal depression, in the manner described by Erlanger and Blair for frog nerve. Recruitment when it occurs must mean either that the period of latent addition is extended with an obliteration of the postcathodal depression, or that a second summation period exists after the postcathodal depression has ended.

The first alternative is unlikely, because in the condition of the nerves in which subthreshold excitability curves are mapped, recruitment experiments should be successful. However, in order to make sure, subthreshold curves were determined on veratrinized frog nerves; and they were found, in confirmation of H. T. Graham (1935), to consist of a period of summation followed by a depression. When the experiment was performed in this way, with single subthreshold conditioning and testing shocks, no definite second period of summation was visible. But the absence of a summation period could not be concluded from this fact, since fundamentally this experiment differs from the situation obtaining during the application of the first two shocks of a train of stimuli only in the size of the shocks employed. Evidently when the stimuli are above the threshold of the most readily excitable fibers, they are so close to the threshold of the next most excitable fibers as to be able to detect the very small enhancement of excitability obtaining in those fibers after the postcathodal depression has ended.

Theoretically there should be a limiting frequency for recruitment in that the interval between the shocks should be longer than the period of postcathodal depression. The hypothesis cannot be tested with trains starting with supraliminal shocks, as the successive components fall within the refractory periods of the preceding responses, causing the fibers responding initially to drop out again; and trains at the start subliminal for all fibers must be employed. In order to demonstrate a limiting frequency it would be necessary to show that a train of subliminal shocks recruiting at one frequency would fail to do so at a higher frequency. But owing to the critical value of the strengths of the shocks which must be employed and the difficulty of maintaining a constant excitability of the preparation, experiments so designed are not very satisfactory. It is found, however, that at frequencies at which successive shocks fall in the periods of postcathodal depression of the preceding shocks, when the first shock is regulated to be just below the threshold, no recruitment appears as the train progresses.

A better argument for a limiting frequency is derived from the indirect evidence contained in some experiments first performed by Lorente de Nó (1937). The essential feature of the experiment is to condition with a train of subthreshold stimuli instead of with a single shock, and to test with a single shock set just above threshold, the cathodes for the stimuli from the two sources being superimposed. A rise or fall of excitability is then revealed by the number of fibers responding. At the end of a train such as this the degree of postcathodal depression is greater than it is after a single conditioning stimulus. Furthermore, if the testing shock is moved into the train, it is found that each interval between the conditioning stimuli is characterized by an initial period of latent addition followed by a

period of postcathodal depression in the pattern of the curve obtained after a single conditioning stimulus.

A still more important feature of the experiment in which a nerve is conditioned by a train of subthreshold stimuli is found in the period following the depression succeeding the last shock. The excitability is so much enhanced during this period that the enhancement can readily be measured by single test shocks. Figure 7 gives an idea of the course taken by the excitability, and it leaves no room for doubt that recruitment must be attributable to a second period of low thresholds. Obviously there are two times at which summation can take place—the classical period of latent addition and a second period starting after the period of postcathodal depression.

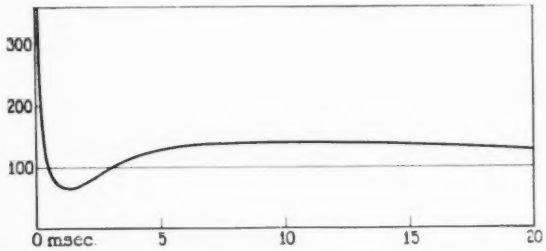


Fig. 7. Excitability of a veratrinized frog nerve following a subthreshold tetanus. Ordinates, height of the testing spike in percentage of the control height. Abscissae, time after the last shock in the conditioning train.

Discussion. All the differences between the records in parts A and B of figure 4 are not explainable on the basis of recruitment. In series B, made on rested nerve, the fibers fail to follow the higher frequencies, although they follow the same frequencies easily in series A. This difference is readily accounted for on the basis of the nature of the after-potentials in the individual components. The negative after-potential is large in series A, whereas it is small in series B; and a large after-potential hastens recovery, shortens the relatively refractory period (H. T. Graham, 1934) and increases the ability to carry a tetanus over a period of time (Gasser, 1937). But while the large after-potential suffices to explain how fibers once responding are able to continue responding, it is powerless to explain how additional fibers may be brought to threshold. The process which brings this about must run entirely subthreshold.

In all the situations in which it has been tried, agents which augment the negative after-potential aid recruitment. They increase the supernormality following the refractory period of the fibers that have responded, and they create a second period of low thresholds following the period of postcathodal depression in the fibers that have not responded. From

this parallelism the most natural conclusion which can be drawn is that the two processes are the same, the only difference being the conditions under which the process is invoked.

When the subthreshold excitability curve of a recruiting nerve is examined it becomes evident that it is made up of an early part comprising the periods of latent addition and postcathodal depression, the time course of which is relatively fixed; and a late period of summation, the duration and degree of which are extremely variable. The contrast here presented reflects the corresponding contrast between the spike and the negative after-potential. The parallelism between the relatively refractory period and the period of postcathodal depression has already been pointed out by Erlanger and Blair. Carrying the parallelism one step further, the periods of latent addition and postcathodal depression taken collectively might be considered to represent the spike process in subthreshold form. The second summation period would then fit into the sequence very well, as the representative of the process which above threshold would produce the negative after-potential.

The two periods of summation in nerve suggest two methods by which summation can be produced in the central nervous system. The first method is very effective and precise in its timing. Two volleys, individually below threshold, in juxtaposition are able to produce a large effect; but the two sets of impulses must arrive almost simultaneously in adjacent endings at the synapses, as shown in the experiments of Lorente de Nó on the oculomotor nucleus, otherwise transmission does not occur. Addition is at its best when the impulses arrive at the same time. After 0.2 msec. the summation begins to fall off rapidly, and after 0.4 msec. it vanishes. All the properties of this mode of summation are clearly those of the early period of latent addition in nerve.

The second type of summation requires no precision in the time of arrival of the separate impulses and takes place slowly. The crossed extension reflex in a spinal animal, as described by Liddell and Sherrington, would be a good example. Evidently each afferent volley arouses but a small increment of excitation, and a large number of additions must be made before the final maximal number of neurons is brought into activity. Recruitment here resembles the recruitment in the nerve experiments where the excitability of the nerve fibers must be progressively built up during the second summation period to a point at which one of the shocks can stimulate; and analogous mechanisms may be involved in the two cases. However, it must not be forgotten that recruitment may also take place by summation of the first type, through properly timed convergence of the sensory impulses, momentarily incident, with impulses impinging upon the same synapses derivative from activity set up in the grey matter by an earlier portion of the sensory bombardment.

SUMMARY

Trains of stimuli below a limiting frequency and at a strength just above threshold for the most excitable fibers of a nerve trunk in suitable nerves are able to recruit fibers over a series of several hundred shocks. Trains starting below threshold may begin to initiate responses after a number of stimuli have been applied.

The recruitment depends upon the fact that the excitability curve following a subthreshold shock is continued after the initial period of latent addition and the subsequent period of postcathodal depression by a second summation period which is variable in extent and duration. A succession of subthreshold shocks progressively increases the excitability of the nerve during this period.

Agents which increase the negative after-potential and the accompanying supernormal responsiveness during an action also augment excitability during the second summation period of subthreshold activity and aid the appearance of recruitment.

Recruitment does not occur in rested mammalian nerves, but it is present if the fibers have recently been active.

REFERENCES

BLAIR, A. E. AND J. ERLANGER. This Journal **117:** 355, 1936.

BREMER, F. AND G. HOMÈS. Arch. Int. de Physiol. **35:** 39, 1932.

CHAUCHARD, A. AND B. CHAUCHARD. Ann. de Physiol. et de Physicochim. Biol. **1:** 6, 1925.

ERLANGER, J. AND E. A. BLAIR. This Journal **99:** 108, 1931–1932.

GASSER, H. S. Excitability cycle, in J. Erlanger and H. S. Gasser. Electrical signs of nervous activity. Philadelphia, Univ. Pa. Press, 1937, p. 183.

GASSER, H. S. AND H. GRUNDFEST. This Journal **117:** 113, 1936.

GRAHAM, H. T. This Journal **104:** 216, 1933.
This Journal **110:** 225, 1934–1935.
This Journal **113:** 52, 1935.

LAPICQUE, L. Ann. de Physiol. et de Physicochim. Biol. **1:** 132, 1925.

LEHMANN, J. E. This Journal **118:** 600, 1937.

LIDDELL, E. G. T. AND C. S. SHERRINGTON. Proc. Roy. Soc. Ser. B. **95:** 407, 1923–1924.

LORENTE DE NÓ, R. This Journal **113:** 524, 1935.

LORENTE DE NÓ, R. Unpublished, 1937.

MONNIER, A. M. L'Excitation Electrique des Tissues. Paris, Hermann and Co., 1934, p. 167.

THE EFFECT OF THE TOTAL REMOVAL OF THE LIVER OF THE MONKEY

STEPHEN MADDOCK[1] AND ANDREA SVEDBERG[2]

From The Surgical Research Laboratory of the Boston City Hospital and the Biochemical Laboratory of Harvard Medical School

Received for publication July 27, 1937

One of the most interesting results of the development of the successful technique for total removal of the liver devised by Mann (1) and his co-workers was the discovery by Bollman, Mann and Magath (2) that in the lower animals destruction of uric acid ceased following hepatectomy. Recently Wakeman and Morrell (3) have reported that monkeys dying with yellow fever failed to show any rise in blood uric acid values although other changes consistent with liver removal occurred. The livers of these animals on pathological examination showed little or no normal appearing tissue (4). These results suggest various possibilities: Either there is some fundamental difference between the purine metabolism of the monkey and that of the dog or the disease process may fail to present a crucial experiment comparable to hepatectomy. In the latter event it might be assumed that there was still sufficient normal liver tissue remaining to carry on some of the functions of the organ; or that the presence of yellow fever masked the effects of liver destruction. It was for the purpose of reaching a decision between these two alternatives that the present experiments were undertaken.

EXPERIMENTAL. Under ether anesthesia, with aseptic precautions, the animals were prepared for liver removal by partial ligation of the portal vein and vena cava as described by Markowitz and Soskin (5) for the dog and later utilized by Drury (6) for the rabbit. In most of the animals a second preliminary operation in which complete ligation of the vessels was accomplished was performed 2 to 4 weeks after the first one. After another interval of 4 weeks or more the operation for total hepatectomy was performed using the technique described by Mann (1) for the dog, except that artificial insufflation was not used. In some of the animals a bilateral nephrectomy was also done at the time of liver removal.

[1] Fellow of the National Research Council, 1928–1930.

[2] Aided in part by a grant from the Ella Sachs Plotz Foundation for Medical Research. Aided in part by a grant from the DeLamar Mobile Research Fund of Harvard Medical School. Read before the Society of Experimental Pathology April 28, 29, 30, 1932, at Philadelphia.

Post-operatively the monkeys were kept on a warm, padded table. To avoid frightening them an anesthesia screen was placed over the table and a tent was made for the animal's head and chest with a sheet. As long as the head was covered the monkeys were usually content to lie quietly, which greatly facilitated handling them, as well as aided in conserving their strength.

TABLE 1

Blood chemical changes following total removal of liver

Monkey 9. Weight 4.0 kgm.

TIME	BLOOD	GLUCOSE	SUGAR	N.P.N.	UREA	AMINO ACID	URIC ACID	CREATININ	SUM OF N	REST N
		grams per kgm.	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*per cent*
Control	I	0.5	130	25	12.5	5.8	0.4	1.4	18 89	24.4
p.m.										
2:10*										
2:50	II	0.5	334	26		6.9	1.5			
3:10	III	0.5	334	27	9.5	7.1	1.9			
4:10	IV	0.5	250	27		7.0	2.1			
5:10*	V	0.5	200	26	10.3	7.3	2.3	1.7	19.1	27.4
6:10*	VI	0.5	148	27		7.8	2.6			
7:10	VII	0.5	114	28	10.6	8.0	2.9			
8:10	VIII	0.5	118	30	10.5	9.4	3.0	2.1	21.6	28.0
9:10	IX	0.5	138	30		10.7	3.2			
10:10	X	0.5	187	34	10.5	11.1	3.3			
11:10	XI	0.5	267	34	10.0	13.7	3.4			
a.m.										
12:10	XII	0.5	188	32		14.8	3.8			
1.10	XIII	0.5			9.6	15.0	4.1			
2.10	XIV	0.5	196	32		17.6	4.4	3.0		
3.10*	XV	0.5	128		10.0	15.8	5.0			
3:45*	XVI	0.5		37	10.5	15.0	4.7			
3:53*	XVII			36	10.5	15.6	5.2	3.3	28.9	19.7
3:55*	XVIII			38	10.4	15.8	5.2			

* Remarks:

2:10 p.m.	Liver removed
5:10	Venous blood
6:10	Venous blood
3:10 a.m.	Animal comatose
3:45	Venous blood
3:53	Right heart. Vomiting and convulsions
3:55	Left heart. Death at 3:58 a.m.

Blood samples were collected directly from the femoral artery or vein through a small incision in the groin made at the time of operation. Glucose was administered in 10 per cent solution into the arm veins in doses of 0.25 to 0.5 gram per kilo per hour.

Urine collections were made in a small metabolism cage prior to opera-

tion. The monkeys were catheterized before placing them in the cage and at the end of the collection period. During the actual experiment urine was collected every 3 to 4 hours. Because of the small amounts obtained post-operatively, these samples could not be analyzed separately, so that the entire amount excreted was pooled and determinations made for the period as a whole.

TABLE 2

Blood and urine changes following total hepatectomy

Monkey 10. 0.5 grams of glucose per kilogram. Weight 2.3 kgm.

Blood

TIME	BLOOD	SUGAR	N.P.N.	UREA	AMINO ACID	URIC ACID	CREATININ	SUM OF N	REST N	REMARKS
		mgm. per cent	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*per cent*	
Control	1	100	28	16.4	6.4	0.3	1.25	23.31	16.7	
p.m.										
2:07										Liver removed
2:25	2	71				0.6				
3:07	3	23		15.2	6.4	0.9		22.32	17.3	
4:07		118	27		6.9		1.28			
5:07	5	93				0.7				
6:07	6	160				0.7				
7:07	7	154	30	16.7	9.9	0.7		27.33	8.8	
8:07	8	182				0.8				
9:07	9	180				0.9				
10:07	1	218		10.8		0.7				
11:07	11	222		7.7	11.8	0.8				
a.m.										
12:07	12	235			13.6	0.9	1.72	22.6	40.5	
12:25	13		38	7.3	14.4	1.1				

Urine

HOURS	TOTAL AMOUNT	AVERAGE OUTPUT	TOTAL N	UREA N	URIC ACID	CREATININ	AMMONIA N	REMARKS
	cc.	*per hour*	*mgm. per hour*	*mgm. per hour*	*mgm. per hour*	*mgm. per hour*	*mgm. per hour*	
25½	100	3.9	9.9	7.4	0.27	2.4	0.18	Before operation. Animal fasting
10	15	1.5	3.2	1.35	0.77	1.1	0.71	After operation

Analytical methods. Filtrates of unlaked blood prepared by the Folin (7) method were analyzed for uric acid and the other non-protein nitrogen constituents. Urine analyses were made by the methods of Folin (8).

RESULTS. The operation for removal of the liver was performed on a series of 25 monkeys, more than half of which lived for 10 hours or longer.

The longest survival was 17 hours. The behavior of the animals following the operation was essentially the same as that described by Mann (9) for the dog except that the excitement stage was limited to a short period of hyperactive reflexes followed by coma.

Figures are presented for 2 typical experiments in tables 1 and 2. Neither of these animals reached the maximum survival period, but the results are more instructive because of the frequent blood samples which were taken in both cases. The blood changes in monkey 9 (table 1) from which the kidneys were not removed but which remained completely anuric throughout the survival period were the same as those found in the nephrectomized animals. It will be noted that uric acid in the blood rose to 13 times its initial value. Urea remained practically constant. The amino acid rise of 272 per cent from the control to the final value is quite striking. Changes in the other substances can be noted by reference to the table.

In table 2 figures are given for the urine chemistry before and after total hepatectomy. Uric acid excretion increased from 0.27 mgm. per hour to 0.77 mgm. per hour—a percentage rise of 350. There is a similarly marked increase in ammonia nitrogen excretion in the urine. The marked drop in urea excretion is mirrored in the reduction in total nitrogen output. Since low urinary output is a common finding after liver removal, unless large amounts of fluid are given, decrease in creatinine excretion can probably be accounted for on this basis.

The changes in the blood in this animal differed somewhat from those found in monkey 9. Neither uric acid nor amino acid nitrogen showed as striking a rise. Urea nitrogen diminished markedly. The terminal rise in non-protein nitrogen was associated with a great increase in undetermined nitrogen in the final bloods.

In addition to the figures given in tables 1 and 2 a few comments may be desirable on the results obtained upon the series as a whole, although the data cannot be presented in detail. In all experiments the changes in blood and urine chemistry were consistent. It was noted, however, that the extent of the chemical changes in animals surviving only a few hours were practically the same as those found where the experiment continued much longer.

The decrease in *blood sugar* was more rapid than that ordinarily noted in dogs (10) (11) or rabbits (12) (13). The average pre-operative level was 88 mgm. per cent, while the average value one hour after operation was 30 mgm. per cent in 8 animals from which data were available. After the administration of glucose to the hypoglycemic animal the return to consciousness was quite as striking as that noted in the dog (10).

The *uric acid* increased from an average pre-operative value of 0.2 mgm. per cent to an average final value of about 2.4 mgm. per cent. In some

instances the rise was as high as 6.0 mgm. per cent. The *urea* content of the blood remained constant, or fell slightly in all anuric or nephrectomized animals. The animals which secreted urine in fairly large amounts showed an appreciable decrease in urea, sometimes to less than half the pre-operative level. The *amino acid nitrogen* increased from an average pre-operative value of 6.4 mgm. per cent to the average final value of 15.1 mgm. per cent. In some cases the terminal value trebled the control figure. Creatinin showed slight rises such as would be expected from nephrectomy alone, from an average pre-operative value of 1.1 mgm. per cent to an average final value of 1.89 mgm. per cent. In the animals which secreted urine there was also a terminal rise in creatinin which, however, never exceeded 0.5 mgm. per cent. The non-protein nitrogen in most animals rose from an average pre-operative level of 30 mgm. per cent to a final figure of 45 mgm. per cent. These rises could not always be accounted for on the basis of nitrogenous constituents, but it was noted that animals in poor condition after hepatectomy showed the greater rise. In view of the striking rises in total creatinin found subsequently in studies on the rabbit (13) it is unfortunate that these values were not obtained in this series of animals.

Bilirubin formation. Following the removal of the liver in the monkey there was an evident accumulation of bilirubin in the blood and tissues which could be noted as early as the 5th hour. The yellow coloration of the subcutaneous fat was more striking than that noted in the blood serum.

The icteric index values reached levels of 6 to 15; the average terminal value for the series was 8.

Van den Bergh reactions were diphasic in all cases where the test was performed.

Corpuscle volume determinations were performed by the hematocrit method in a few of the animals. In each instance there was a decrease in cells from a pre-operative level of 42–44 per cent to 30–32 per cent. In no case did the cell volume fall below 28 per cent.

Discussion. In general the monkey behaves like the dog (10) (11) and rabbit (12) (13) following total hepatectomy and the chemical picture is so consistent with the results of other workers that no additional comment is necessary except for the behavior of uric acid. Blood uric acid rises quickly and generally reaches a level of 10 to 12 times the pre-operative value, while concomitant with this rise there is a great augmentation of the uric acid output in the urine, indicating that the site of the destruction of this substance has been lost. This is an interesting finding in the light of the yellow fever experiments of Wakeman and Morrell (3) who found no rise of uric acid (less than 1.0 mgm. per cent) in animals dying of the disease. It is apparent that even though histologically no normal looking liver tissue remains there may still be a sufficient number of cells present

capable of maintaining some of the functions of the organ, such as the destruction of uric acid.

It seems possible that a similar situation might occur in the human dying of acute yellow atrophy of the liver, in which condition low terminal uric acid values in the blood are usually found (14) (15).

SUMMARY

1. Data are presented on a series of 25 monkeys following total hepatectomy.

2. The monkey tends to survive liver removal about the same length of time as does the dog.

3. The typical features of the blood chemical picture are: The blood sugar falls; the urea remains constant in nephrectomized, or anuric animals, and falls in animals which excrete urine; uric acid, amino acid and creatinine all increase.

4. The urinary output following hepatectomy in the monkey shows: Decreased secretion; decrease in total N and urea; increase in amino acid N, uric acid, and ammonia N.

5. The rise in blood uric acid and its increased output in the urine observed in hepatectomized animals is in contrast to the findings of Wakeman and Morrell (4) in yellow fever. This suggests that very small amounts of functionally normal liver tissue may have persisted in yellow fever livers even though normal liver cells were not noted microscopically.

6. It is suggested that a similar situation may exist in human patients dying of severe liver damage.

REFERENCES

(1) Mann, F. C. Am. J. Med. Sc. **161:** 37, 1921.
(2) Bollman, J. L., F. C. Mann and T. B. Magath. This Journal **72:** 629, 1925.
(3) Wakeman, A. M. and C. A. Morrell. Arch. Int. Med. **46:** 290, 1930.
(4) Hudson, N. P. Am. J. Path. **4:** 407, 1928.
(5) Markowitz, J. and S. Soskin. Proc. Soc. Exper. Biol. and Med. **25:** 7, 1927.
(6) Drury, D. R. J. Exper. Med. **49:** 759, 1929.
(7) Folin, O. J. Biol. Chem. **86:** 173, 1930.
(8) Folin, O. A laboratory manual of biological chemistry. D. Appleton Century Co., New York, 1934.
(9) Mann, F. C. Medicine **6:** 419, 1927.
(10) Mann, F. C. and T. B. Magath. Arch. Int. Med. **30:** 73, 1922.
(11) Maddock, S. and H. C. Trimble. The effect of total hepatectomy on the dog. (Unpublished.)
(12) McMaster, P. D. and D. R. Drury. J. Exper. Med. **49:** 745, 1929.
(13) Svedberg, A., S. Maddock and D. R. Drury. In press.
(14) Rabinowitch, I. M. J. Biol. Chem. **83:** 333, 1929.
(15) Maddock, S. Personal observations.

THE EFFECT OF TOTAL REMOVAL OF THE LIVER IN THE RABBIT[1]

ANDREA SVEDBERG, STEPHEN MADDOCK AND DOUGLAS D. DRURY

From the Laboratory of Biochemistry of Harvard Medical School, the Surgical Research Laboratory of the Boston City Hospital and the Collis P. Huntington Memorial Hospital

Received for publication July 27, 1937

The clinical effects of total hepatectomy in the rabbit were first described by Drury (1) in 1929. At the same time McMaster and Drury (2) published the results of a study on rabbits in which marked liver insufficiency had been produced surgically. In the latter paper data were presented graphically for sugar, urea and uric acid which closely paralleled the changes noted by Mann and his co-workers (3, 4, 5) on dogs. Since, however, no blood chemistry figures were given for the animals in which total removal of the liver was performed, the present investigation was undertaken, in order to furnish this information and also to present more complete analyses of the nitrogenous constituents of the blood.

EXPERIMENTAL. The experiments were of two types: *a*, hepatectomy with or without nephrectomy, and *b*, hepatectomy with removal of all the intra-abdominal viscera.

The operative technique used for hepatectomy was that described by Drury (1). An interval of 4 to 6 weeks was allowed to elapse between the preliminary operation and the hepatectomy. All animals were fasted for 96 hours prior to removing the liver, but even with this precaution a certain amount of food always remained in the stomach.

The operative procedure for the evisceration experiments was similar to that described by Mann (6) for the dog, in which after a preliminary sub-total ligation of the vena cava all the intra-abdominal viscera were removed except the kidneys and about 3 cm. of the terminal end of the rectum.

Following hepatectomy with either type of operation micro blood sugars (7) were taken hourly and glucose administered intravenously in 10 per cent solution.

Chemical methods. All analyses were made by Folin's methods for unlaked blood (8).

[1] Aided in part by a grant from the DeLamar Mobile Research Fund of Harvard Medical School and the Ella Sachs Plotz Foundation for Medical Research.

RESULTS.[2] The operation for total removal of the liver was successfully performed on 17 rabbits. In 10 of the animals the kidneys were also removed. The other 7 were not nephrectomized but in only 3 cases was there any secretion of urine. The average length of life for the series was 15.6 hours; 5 animals lived over 20 hours. Total abdominal evisceration was performed on 4 animals. A bilateral nephrectomy was done on 2 additional control animals.

The post-operative behavior of the hepatectomized animals corresponded in all respects to the description given by Drury (1). The fall in blood sugar so prominent and early a feature in all species following liver removal occurred in every case. During the early hours a dosage of 150 mgm. per kilo was usually sufficient to maintain the blood sugar at normal levels. Later the dosage had to be increased, but it was never necessary to give

TABLE 1

Values of the nitrogenous constituents of unlaked blood in normal rabbits

ANIMAL NUMBER	URIC ACID	UREA N	AMINO ACID N	CREATININE		N.P.N.	UNDETERMINED N IN N.P.N.
				Preformed	Total		
							per cent
71	0.27	19	5.4	1.3	1.5	26	4
89	0.34	24	8.7	1.2	1.9	38	13
13	0.30	21		1.3	2.4	38	
8	0.29	21		1.3	2.0	39	
86	0.17	15	7.8	2.1	2.4	27	12
94	0.19	18	9.1	1.8	2.6	32	11
93	0.26	16	8.5	1.8	2.4	30	16
96	0.33	15	9.5	1.0	1.8	27	6
Average..	0.27	18 6	8.1	1.5	2.1	33	10

more than 500 mgm. per kilo. In the late stages the amount of glucose could sometimes be reduced without hypoglycemia developing. No figures for glucose determinations are given in this paper because of the great regularity of this well established phenomenon.

Because of the fact that unlaked blood was used for the determinations table 1 has been inserted for the purpose of comparison with the results of previous workers. The difference between these values and the ones obtained by the Folin-Wu method on whole blood are mainly: Uniformly low uric acid values, close approach of the total creatinine to the preformed creatinine values, smaller amount of undetermined nitrogen, and lower amino acid nitrogen values due to the elimination of the glutathione (10), and ergothionin (11) of the corpuscles.

[2] Some of these results have already been partially presented in connection with a discussion of the undetermined nitrogen of the blood (9).

In table 2 results are given for a typical experiment in which both the liver and kidneys had been removed. The constant urea level is quite striking. The rises in uric acid and in amino acid nitrogen are similar to those noted in other species. Preformed creatinine shows an increase corresponding to the findings after nephrectomy alone; total creatinine, however, shows a great augmentation as does also the non-protein nitrogen. It will be noted that the percentage of undetermined N was markedly increased in the terminal specimens.

In table 3 (a) the data for one of the animals which secreted some urine are included. In this instance there is a definite fall in urea, with moderate rises in both uric acid and amino acids. The creatinine figures, both preformed and total, contrasted sharply to those found in rabbit 91 (table 2). These values as well as the non-protein nitrogen are much lower

TABLE 2

Total hepatectomy and nephrectomy

Rabbit 91, June 2, 1930

BLOOD NUMBER	TIME	URIC ACID	UREA N	AMINO ACID N	CREATININE		N.P.N.	UNDETERMINED N IN N.P.N.
					Preformed	Total		
	hours							*per cent*
1	Pre-op.	0.42	20	8.1	1.6	2.9	40	27
2	6	2.7	20	14.8		6.3		
3	12	3.7		15.4		9.0	61	34
4	16	4.7	19		4.8	14.0		
5	19	5.1		17.4		17.0		
6	23	5.5	21	17.7		19.0	79	40
7	26	7.4	21	19.0	5.7	23.0	94	46

The very low percentage of undetermined nitrogen (8 per cent) in the final value is also worthy of note.

Table 3 (b) gives the values of the terminal specimens for 2 of the eviscerated animals. It is unfortunate that earlier specimens are not available for these experiments. The remarkable features of these results are the very high amino acid values, the high creatin and non-protein nitrogen figures, and also the low percentage of undetermined N.

Since no figures seem to be readily available for rabbits in which nephrectomy alone has been performed, table 4 has been included for the purposes of comparison with the liverless animals.

Discussion. The most striking feature of these experiments is the sharp rise in creatin. The average value for normal animals is 0.6 mgm. per cent, whereas the average value for 12 hepatectomized animals living an average of $15\frac{1}{4}$ hours was 11.0 mgm. per cent. The average value for 4 totally eviscerated animals at death (average 35 hours) was 54 mgm. per

cent. It is possible that all of the substance determined as creatin after hydrolysis at 130°C. is not actually creatin but some other diffusible nitrogenous material of tissue origin, but the Folin (8) method is undoubtedly much more specific when used on the unlaked filtrate. This is evidenced by the low values before operation (table 1), where the total creatinine averages only 1.0 mgm. per cent higher than the preformed creatinine.

The large increase in creatin suggests that a breakdown of tissue material is taking place, liberating this substance into the blood stream. That

TABLE 3

BLOOD NUMBER		TIME	URIC ACID	UREA N	AMINO ACID N	CREATININE		N.P.N.	UNDETERMINED N IN N.P.N.
						Preformed	Total		
(a) Total hepatectomy, kidneys intact									
		hours							*per cent*
79	1	Pre-op.	0.14	18.6	11.1	1.5	2.5	33	7.4
	2	17	2.8	17.4	23.3		6.0	43	
	3	20½	6.0	12.5	24.7	1.88	13.5	52	8.3
51	1	Pre-op.	0.36	23.0	9.0	1.25	2.2	34	3.0
	2	15½	5.2	23.0	23.0		18.8	91	40.0
	3	18	5.8	24.0	24.4	4.3	20.9	93	38.0
8	1	Pre-op.	0.29	21.4	7.8	1.33	2.0	39	23.0
	2	10	5.7	21.0	24.6	2.92	13.3	79	33.6
(b) Intra-abdominal evisceration with hepatectomy, kidneys intact									
92	1	24	2.0	27.0	63.0	5.0	55.0	127	12.0
	2	32				5.2	79.0	154	
12	1	28	1.7	20.0	51.0	4.5	27.0	90	9.0
	2	34	2.4	20.0	61.0	5.1	46.0	123	19.0

creatin is a part of the active living protoplasm was pointed out by Folin and Dennis in 1914 (12). This view is further supported by the recent work of Fiske and Subbarow (13) who have proven that phospho-creatin is an important muscle constituent. Since the animal devoid of abdominal viscera consists much more largely of muscle this might tend to accentuate the process of the breakdown of phospho-creatin, possibly accounting for the unusually high values in these animals.

There is naturally an increase in non-protein nitrogen in the rabbit corresponding to the high amino acid and creatin values. The dog (14) and the monkey (15) rarely show a rise of more than 10 mgm. per cent in non-

protein nitrogen throughout the course of the experiment. At first it was thought that the figures in the rabbit were indicative of venous stasis in the gastro-intestinal tract but the animals in which all the intestines were removed show an even more striking rise. Accompanying this rise in N.P.N. there is also a definite increase in the percentage of undetermined nitrogen of the N.P.N. The hepatectomized animals show an average difference between the percentage of undetermined N before operation and at death of 19.7 per cent (10 per cent to 35 per cent), whereas after nephrectomy alone the rise in undetermined N after 24 hours is only 3 per cent. This is less striking in the animals which were eviscerated, where there is practically no change despite the very high non-protein nitrogen values. This would seem to indicate that many of these unknown nitrogenous

TABLE 4

Nephrectomy

BLOOD NUMBER	TIME	URIC ACID	UREA N	AMINO ACID N	CREATININE		N.P.N.	UNDETERMINED N IN N.P.N.
					Preformed	Total		
								per cent
Brown	June 2							
1	Pre-op.	0.34	24	8.7	1.2	1.9	38	13
2	6 hrs.	0.34	32	5.9		2.4	46	15
3	14 hrs.	0.43	37	5.8		2.9		
4	18 hrs.	0.29	40	5.3			54	
5	June 3				8.6		93	
6	June 4	0.4	78	5.2		10.5	103	16
7	June 5		102	6.2	9.6	15.4	147	22
8	June 6	0.6	147	7.6	12.8	18.2	199	16
9*	June 7	0.4	133	5.4	9.2	13.8	165	13
10	June 9	1.1	250	8.9	8.5	32.0	358	22

* Animal drank 200 cc. of water shortly before this blood was taken.

products actually come from the gastro-intestinal tract. Their absence might account for the consistently longer life of the eviscerated animals.

SUMMARY

Data are presented on a series of 17 rabbits following total hepatectomy, with and without nephrectomy. Some experiments have been made on animals with total abdominal evisceration including liver removal.

The rabbit tends to survive liver removal longer than the other animals studied.

The most characteristic chemical features of the blood picture are: The blood sugar falls. Urea remains constant or falls slightly. Uric acid, amino acids and creatin increase. The increase in amino acid and in

creatin is more marked in eviscerated animals. The hepatectomized animals show a considerable rise in undetermined N.

REFERENCES

(1) DRURY, D. R. J. Exper. Med. **49:** 759, 1929.
(2) MCMASTER, P. D. AND D. R. DRURY. J. Exper. Med. **49:** 745, 1929.
(3) MANN, F. C. AND T. B. MAGATH. Arch. Int. Med. **30:** 73, 1922.
(4) BOLLMAN, J. L., F. C. MANN AND T. B. MAGATH. This Journal **69:** 371, 1924.
(5) BOLLMAN, J. L., F. C. MANN AND T. B. MAGATH. This Journal **72:** 629, 1925.
(6) MANN, F. C. Ergebn. d. Physiol. **24:** 379, 1925.
(7) FOLIN, O. J. Biol. Chem. **77:** 421, 1928.
(8) FOLIN, O. J. Biol. Chem. **86:** 173, 1930.
(9) SVEDBERG, A. Nordisk, Medicinisk Tidskrift **3:** 241, 1931.
(10) HOPKINS, F. G. Biochem. J. **15:** 286, 1921.
(11) BEHRE, J. A. AND S. R. BENEDICT. J. Biol. Chem. **82:** 11, 1929.
(12) FOLIN, O. AND W. DENIS. J. Biol. Chem. **17:** 487, 1914.
(13) FISKE, C. H. AND Y. SUBBAROW. J. Biol. Chem. **81:** 629, 1929.
(14) MADDOCK, S. AND H. C. TRIMBLE. Unpublished data.
(15) MADDOCK, S. AND A. SVEDBERG. This Journal.

THE ABSORPTION SPECTRA OF VISUAL PURPLE AND ITS PHOTODECOMPOSITION PRODUCTS

A. C. KRAUSE AND A. E. SIDWELL, JR.

From the Division of Ophthalmology, Department of Surgery and the George Herbert Jones Chemical Laboratory, the University of Chicago

Received for publication July 27, 1937

The study of the chemical and physical properties of visual purple, a photosensitive substance which occurs in retinal rods, has been the subject of numerous investigations since its discovery by Krohn (1) in 1842. An extensive literature (2) has dealt with the kinds, occurrence and properties of visual purple, with the mechanism and products of its decomposition by light, heat and chemical reagents, and with the problem of its regeneration. Great interest in problems concerned with visual purple has developed with the increase of applicable experimental methods.

The purpose of this investigation was to continue a study of the chemistry of the photosensitive substances of the retina. For this purpose an accurate photoelectric spectrophotometer, adapted for use in the ultraviolet and visible regions of the spectrum, was employed to determine the spectroscopic properties of visual purple, visual yellow and visual white. The results of this study, together with those of other investigators, are considered in this discussion.

Experimental procedure; photoelectric spectrophotometer. The absorption spectra considered in this paper were determined by means of a photoelectric spectrophotometer,[1] the details of the construction, calibration and accuracy of which have been described by Hogness, Zscheile and Sidwell (3). The apparatus was specially designed to facilitate rapid interchange of the optical parts necessary for work in the visible and ultra-violet regions of the spectrum.

Carefully matched absorption cells with quartz windows are placed behind the exit slit of the monochromator in this apparatus, so that solutions are exposed to only about 10^{-8} lumens. As a consequence of the low light intensities employed, photochemical decomposition during the course of the determination is negligible. This fact was determined for solutions of visual purple by repeated measurements of transmission at various wave lengths during the course of any single experiment. The

[1] Spectroscopic work with this apparatus is supported by a grant from the General Education Board.

apparatus allows the use of narrow slits and consequently narrow spectral regions isolated by the monochromator at any given wave length, thus increased accuracy in the determination of the relative proportions of each curve was obtained. The wave length limits of the radiation passing the second slit of the monochromator, the spectral region isolated, used in the determination of the spectra reported here are given in table 1. No filters were employed.

The spectra reported here were determined by comparison of a given solution with a quantity of the respective solvent or diluent used in the preparation of that solution. The values of the relative intensities of light of a given wave length transmitted by the solvent and by the solu-

TABLE 1

Spectral regions isolated for various wave length regions studied

WAVE LENGTH REGION	OPTICAL PARTS	SLIT WIDTH EMPLOYED	SPECTRAL REGION ISOLATED
A.U.		*mm.*	*A.U.*
2400–2800	Crystal quartz-rock	0.10	6.6–11.0
2800–3400	salt	0.05	5.5–12.5
3400–4000		0.03	7.5–12.5
4000–4150		0.05	5.2– 6.0
4150–4300	Glass	0.03	3.6– 4.0
4300–4600		0.02	2.7– 3.1
4600–6000		0.01	2.3– 4.5

tion, as measured by galvanometer deflections, were related by Beer's law in the form of equation (1).

$$\log_{10} \frac{I_0}{I_x} = \alpha C l = \alpha' c' l' \qquad (1)$$

In this equation,

I_o = relative intensity of radiation passing through solvent
I_x = relative intensity of radiation passing through solution
α = specific absorption coefficient
C = concentration of absorbing material (grams/liter)
l = length of absorption cell (one centimeter in all experiments reported here)
α' = relative absorption coefficient
c' = relative concentration of absorbing material.

Since the absolute concentration of the absorbing material, C, for the solutions was unknown in most cases, the relative values of $\log_{10} \frac{I_o}{I_x}$ were plotted versus wave length. When it seemed advisable to weigh the

material placed in a given solution the specific absorption coefficients were calculated and plotted versus wave length.

Preparation of visual purple solutions. The solutions of visual purple used in the various spectroscopic experiments to be described were prepared from retinas obtained from the eyes of freshly slaughtered cattle. The technique of dissection was such that within three hours after removal from the animals, 200 to 300 retinas could be prepared for the extraction process. Dark adaptation of the eyes, dissection of the retinas and subsequent manipulation of the extracted materials were carried out in a dark room. A 10 watt ruby lamp served to illuminate the dissection table and the extraction apparatus. The extraction of visual purple from the retinal tissue was carried out as follows:

Two hundred retinas (wet weight 121 to 132 grams) were ground together with 250 cc. of cold (4° to 8°C.) 1 per cent sodium chloride solution in a mortar. The suspension thus obtained was placed in a centrifuge tube which was then maintained at 2000 r.p.m. for twenty minutes. During the process of centrifugation the suspended material separated into three distinct layers. The top layer of supernatant liquid was removed by decantation. The middle layer, rich in visual purple, was carefully decanted and scraped from the surface of the bottom layer with the aid of a flattened glass rod. The bottom layer was discarded.

The middle layer which contained visual purple, was washed twice with 50 cc. portions of 1 per cent sodium chloride solution. The red residue was then treated with 30 cc. of 2 per cent sodium glycocholate solution which contained 1 per cent of sodium chloride. The mixture was triturated from time to time and allowed to extract for at least two hours in the cold. The mixture was transferred to a centrifuge tube and was allowed to separate at 2000 r.p.m. for twenty minutes. The red supernatant liquid was decanted and the residue was reëxtracted with two 10 cc. portions of the mixture of 2 per cent sodium glycocholate and 1 per cent sodium chloride solution.

The combined extracts were adjusted to a total volume of 50 cc. Twelve grams of anhydrous sodium sulfate were then added to the solution and after one hour a red precipitate was filtered off. This precipitate was redissolved in 50 cc. of distilled water to produce a solution which was passed through a paper filter, then centrifuged to remove shreds of paper. The solution of visual purple thus obtained was then ready for spectroscopic study. Solutions prepared in this way were optically clear and generally required a dilution of one volume of solution to five volumes with the mixture of 2 per cent sodium glycocholate and 1 per cent sodium chloride solution for optimum transmission values in the visible region.

Preliminary preparations of visual purple contained extraneous material which produced undesirable turbidity. It should be emphasized that

solutions of visual purple for spectroscopic study must be optically clear. Solutions which satisfied this condition and for which Beer's law was obeyed may be prepared as described.

Absorption spectrum of visual purple. To determine the absorption spectrum, a portion of a freshly prepared solution of visual purple was transferred, in a dark room, to one of a pair of cleaned and optically balanced absorption cells. The other cell was filled with a portion of the diluent. The filled cells were transferred to the dark box of the absorption apparatus, and the transmission of the solution of visual purple was measured at the wave length of maximum absorption. From these data, dilutions of the original solution were prepared with 2 per cent sodium glycocholate and 1 per cent sodium chloride solution in order that each portion of the spectrum might be determined with solutions for which the transmission varied only slightly from 50 per cent. Measurements of absorption were made at 50 A.U. intervals except in the regions of maxima.

The upper curve in figure 1 and the solid circles in figure 2 illustrate the absorption spectrum of visual purple from 4000 A.U. to 6000 A.U. and from 2400 A.U. to 6000 A.U. respectively.

Absorption spectrum of visual yellow. A solution of visual purple (that which gave rise to the absorption spectrum shown by the line drawn through the solid circles in fig. 2) was bleached by exposure to white light (60 watt Mazda lamp at 50 cm.) for thirty minutes. The absorption curve determined from the study of the yellow solution obtained is shown in figure 2 (open circles). Slight turbidity developed during the process of bleaching this and all other aqueous solutions of visual purple.

Absorption spectra of partially bleached solutions of visual purple. To study the relation between visual purple and visual yellow the absorption spectrum of a solution of visual purple was first determined in the visible region as described above. This solution was allowed to remain in the absorption cell which was next exposed to light from a 60 watt Mazda lamp at a distance of 50 cm. for one minute. The absorption spectrum of the solution was rapidly obtained. The solution was then exposed to the radiation from the lamp for one minute more, followed by rapid redetermination of the absorption spectrum. This process was repeated so that the solution was exposed to light for a total of twenty minutes. Figure 1 illustrates the absorption spectra obtained after exposure of a solution of visual purple for 1, 2, 4, 8 and 12 minutes respectively. Exposure for twenty minutes produced no detectable change in the curve as obtained after 12 minutes of exposure to the lamp.

Absorption spectrum of material extracted from bleached aqueous solutions of visual purple. A solution of visual purple was bleached and the resulting yellow solution was extracted with an equal volume of low boiling (30° to

60°C.) petroleum ether. This extract was filtered and dried with anhydrous sodium sulfate. After decantation from the sodium sulfate, the solution was centrifuged and finally the petroleum ether was removed by

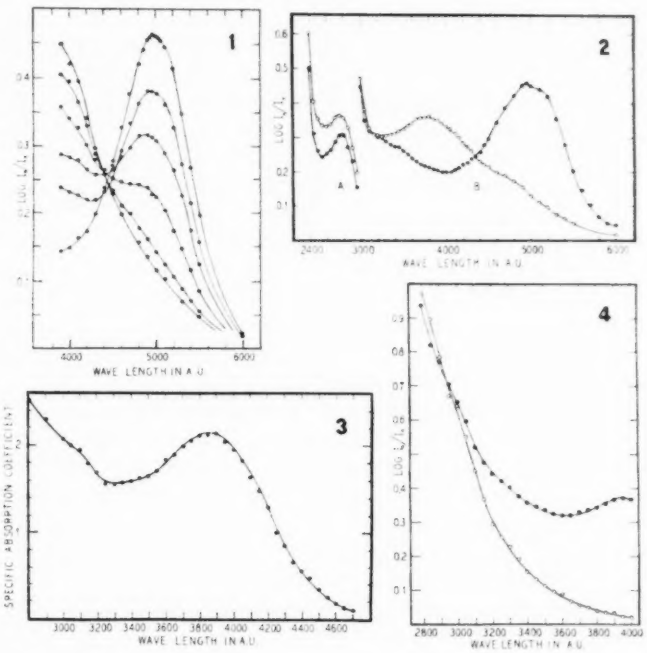


Fig. 1. The effect of partial bleaching upon the absorption of a solution of visual purple. The top curve on the right represents the absorption curve of the original unbleached solution of visual purple. The lower curves in descending order represent respectively the absorption spectra of visual purple after 1, 2, 4, 8 and 12 minutes' exposure to light.

Fig. 2. The upper right curve represents the absorption spectrum of visual purple. The lower curve represents the absorption spectrum of visual yellow after the visual purple is bleached into visual yellow.

Fig. 3. The curve of the absorption spectrum of the golden yellow pigment which was extracted from visual purple.

Fig. 4. The upper curve shows the absorption spectrum of visual yellow and the lower curve of visual white.

evaporation. The yellow residue was re-dissolved in chloroform for spectroscopic examination. Characteristics of the absorption spectrum of this material are shown in figure 3.

The data plotted in figure 3 were obtained from a weighed portion of

yellow material obtained by petroleum ether extraction of a bleached solution of visual purple. This material had been absorbed from petroleum ether solution upon calcium hydroxide (Merck) in a glass tube arranged for chromatographic analysis. The single yellow colored layer obtained in the chromatogram was removed from the tube. The yellow pigment was eluted from the calcium hydroxide by means of a five per cent solution of methyl alcohol in petroleum ether. The solution obtained was filtered, centrifuged and finally the yellow material was obtained by evaporation of the solvent. A fraction weighing 20 mgm. contained all of this yellow pigment from 300 cattle retinas. A weighed portion of the material was dissolved in chloroform for spectroscopic study.

The absorption spectrum of visual white. A portion of a chloroform solution of visual yellow pigment was exposed to direct sunlight in an absorption cell for one hour. The absorption spectrum of the colorless solution thus obtained was determined. The absorption spectrum of a second portion of the original solution of the pigment was determined under the same conditions of dilution for direct comparison. The absorption curves obtained are shown in figure 4.

Discussion. The absorption spectra of purified solutions of bovine visual purple were found to have a maximum at 4950 A which was a characteristic of twelve different preparations of visual purple (double open circles in fig. 1 and black discs in fig. 2). The curve for visual purple (fig. 2) also exhibits a maximum at 2750 A, and a definite minimum near 4000 A. The position of the maximum in the visible region is in qualitative agreement with the work of others, although it is believed that the use of narrow spectral regions isolated by the monochromator allows the claim of high accuracy in its determination. It can be shown that the use of light filters, or of excessively wide wave length ranges from a monochromator, does not permit a detailed study of absorption in the neighborhood of a maximum or minimum.

The effect of light on visual purple solutions is shown in figure 1. It may be inferred that as light acts upon visual purple, visual yellow is formed. The characteristic end absorption curve of visual yellow in the visible region is shown by the upper curve at 4000 A in figure 1. The changes found in the absorption spectrum of visual purple upon exposure to light are in general agreement with the results of Hosoya and Zempei (4), who studied the visual spectrum. A study of absorption in the ultraviolet reveals an absorption maximum occurring at 3800–3900 A in the curve for visual yellow solutions (open circles, fig. 2). This characteristic absorption maximum indicates the presence of a distinctly different molecular species produced by rapid photo-decomposition of visual purple. This spectrum, taken in conjunction with the fact that the position of the maximum at 4950 A undergoes no shift as the usual purple bleaches,

indicates that visual purple in light forms only one colored decomposition product, namely, visual yellow. In this connection the excellent study by Dartnell, Goodeve and Lythgoe (5) of the bleaching of visual purple should be mentioned. These workers found agreement between their experimental data and a mathematical treatment based upon the theory of the photochemical decomposition of an absorbing substance by a mechanism which does not involve absorbing (colored) intermediates. The data of figure 1 for the wave length 4950 A may be related by an equation similar to that given by Dartnell, Goodeve and Lythgoe, namely,

$$\log \frac{I_t}{I_f - I_t} = mt = c$$

where I_t = intensity of light passing through solution at time t,
I_f = intensity of light passing through solution at completion of photochemical reaction,
t = time of exposure to light,
and m and c are constants.

The long wave portion of the characteristic absorption band of visual yellow (open circles, fig. 2) at 3800–3900 A accounts for the light yellow color of such solutions. The absorption band at 2750 A is similar to that found for visual purple (at A on the left of fig. 2 plotted $\frac{1}{5}$ scale). This band is characteristic of proteins. The striking difference in the longer ultra-violet is of great interest. The absorption curve of the isolated purified yellow substance which colors visual yellow solutions, and which may be removed from visual purple (5) and visual yellow solutions by appropriate treatment, is recorded in figure 3. This curve corresponds with the absorption spectrum of visual yellow when wave lengths are considered.

Evidently visual purple is protein combined with a prosthetic group. Whether the change in spectrum is brought about by a change in the structure of the prosthetic group, or is a result of simple cleavage of protein from such a group is an open question. It is logical to assume that a change in the conjugation of bonds within the prosthetic group may bring about cleavage.

There are other reasons to believe that bovine visual yellow is a single substance. Isolated visual yellow, also called the golden yellow lipid (6), before and after exposure to mild alkaline treatment to hydrolyze any ester of the substance, gave only one ring in chromatographic absorption tubes filled with calcium hydroxide, calcium carbonate and aluminum oxide. Upon washing with solvent no separation into layers could be detected. Further, the spectro-photometric measurements of visual yellow obtained by various procedures gave identical curves. For these reasons it is probably premature to speculate on the nature of the yellow

substance of visual yellow solutions until further studies yield evidence of its chemical identity.

The absorption curves of a solution of extracted and purified visual yellow before and after exposure to direct sunlight are shown in figure 4. The drop in absorption illustrated by the lower curve accounts for the colorless solutions obtained by strong illumination. The lower curve is probably that of visual white, previously believed to be an artifact due to the study of extremely low concentrations of visual yellow. Although the region studied here yielded no characteristic bands for this substance (or mixture of substances), further study may lead to the isolation of the end products of the decomposition of visual yellow.

The absorption spectra of solutions and lipid extracts of retina from which the bacillary layer was completely removed gave no evidence of the presence of visual purple or yellow. There is good reason to believe visual purple is entirely in the bacillary layer of the retina.

In the purified visual purple solutions no evidence of vitamin A was found spectrophotometrically. Vitamin A from whole retina was prepared according to the method of Wald (7). If the generally accepted methods are applied, a maximum quantity of about 0.5 gamma of vitamin A per retina was obtained. Most of the vitamin A was found in non-bacillary layer of the retina. Difference in the spectrophotometric curves (not given) of vitamin A from dark adapted retina and from retina exposed to sunlight showed that the amount of vitamin A decreased in sunlight.

No vitamin A was found in the decomposition products of visual purple, visual yellow, and the end colorless substance. Although particular attention was given to vitamin A, no evidence was found for the assumption of Wald (7) that visual purple upon exposure to light liberated or formed vitamin A. The visual yellow behaves like an unsaturated compound of the hydrocarbon type. However, it cannot be called a carotenoid merely on the basis of a color reaction, which has been shown to be nonspecific, and of a comparison with properties of astacene.

SUMMARY

Bovine visual purple is lipid conjugated with a protein. Upon exposure to light there occurs a photochemical reaction which results in the splitting of visual purple into protein and a colored lipid called visual yellow, or golden yellow lipid. Further exposure to light or heat changes the colored lipid into a colorless lipid. Evidently, vitamin A is not formed by the photochemical reaction of visual purple or of visual yellow.

The experimental procedures were performed with the aid of Mr. W. Tauber.

REFERENCES

(1) KROHN, A. Nov. act. akad. Caesar Leopoldino-Carolinse **19:** 1. 1842.
(2) KRAUSE, A. C. The biochemistry of the eye. Johns Hopkins Press, Baltimore, 1934a.
Arch. Ophth. **15:** 522, 1936b.
(3) HOGNESS, T. R., F. P. ZSCHEILE AND A. E. SIDWELL, JR. J. Physiol. Chem. **41:** 379, 1937.
(4) HOSOYA, Y. AND S. ZEMPEI. Tohoku J. Exper. Med. **27:** 172, 1935.
(5) DARTNELL, H. J. A., C. F. GOODEVE AND R. J. LYTHGOE. Proc. Roy. Soc., London, A **156:** 158, 1936.
(6) KRAUSE, A. C. Arch. Ophth. (in press).
(7) WALD, G. J. Gen. Physiol. **19:** 351, 1935.

THE ADRENALS AND GONADS OF RATS FOLLOWING THYROIDECTOMY CONSIDERED IN RELATION TO PITUITARY HISTOLOGY[1]

ISOLDE T. ZECKWER

From the Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia

Received for publication July 27, 1937

When the pituitary is greatly altered in its cellular make-up, both quantitatively and qualitatively, by means of thyroidectomy, it is to be expected that altered functional changes would result from the altered structure. Many of the studies of the histological changes in the pituitary resulting from thyroidectomy were made before the present knowledge of the physiology of the pituitary had developed, and so in the past it was impossible to interpret the significance of the morphological changes. Some of the literature has been reviewed in a recent morphological study (7). Briefly, the changes in the rat consist of a marked loss of acidophiles, and accumulation of large amounts of hyaline material within cells which have fine granules and which have been considered by some workers as chromophobes and by others as basophiles. The present author in studying pituitaries of rats that had been both thyroidectomized and gonadectomized (10) regarded these thyroidectomy cells as entirely different from castration cells in many histological details, and possibly derived from another cell than that giving rise to the castration cell. The blue appearance of the cell is dependent more upon the blue staining of the hyaline material in which the cytoplasmic granules are scattered than on the staining of the granules themselves. In spite of the loss of acidophiles the pituitaries are greatly increased in weight due to the abnormal cells and hyaline material.

With such structural changes in the pituitary, one might expect that the production and storage of its various hormones would be altered. In recent experiments the present author has studied the thyrotropic hormone content of such pituitaries (8) by means of injecting the whole pituitaries into guinea pigs. No biological assay is at present practical for the determination of the adrenotropic content of such small pituitaries as those of the rat. However, the adrenal weights of thyroidectomized rats when

[1] Aided in part by grants from the National Research Council and from the Faculty Research Committee, University of Pennsylvania.

compared with weights of adrenals of non-thyroidectomized rats could be regarded as a measure of the adrenotropic activity of the thyroidectomized rat's pituitary. Similarly weights of gonads of such animals could be regarded as a measure of the gonadotropic activity of the pituitary. In the case of the gonadotropic hormone, biological assay has been made of the pituitaries from female thyroidectomized rats but not from males. Evans and Simpson (2) found that the pituitaries of female thyroidectomized rats had less gonadotropic hormone. Hohlweg and Junkmann (4) implanted pituitaries of female thyroidectomized rats into infantile female rats. No estrus resulted. Smith and Engle (5) found no difference in gonadotropic effect between normal and thyroidectomized rats' pituitaries, 200 days after thyroidectomy. Similarly Van Horn (6) found no change in gonadotropic effect on implanting pituitaries from thyroidectomized female rats five weeks after operation.

There are of course numerous weights of organs of thyroidectomized animals recorded in the literature. Fifty articles have been published by Hammett from 1920 to 1927 on the weights of various organs of thyroparathyroidectomized rats, and he reviewed the earlier literature. Hammett (3) stated that "the initiation of a thyroid deficiency at any time before the pubertal adjustment is in full swing (65 days of age) brings about a retardation in adrenal growth which parallels in direction the change in body weight with change in age at time of thyroid removal." . . . "The factors which come into play at puberty disrupt the simple dependence of the adrenals on the effectiveness of the growth processes of the body as a whole, and turn the organ over to the thyroid." . . . "The growth of the adrenals in the sexually mature albino rat is specifically dependent on thyroid function."

The object of the present experiments was to study the effect of thyroidectomy on the growth of adrenals and gonads as contrasted with visceral growth, and then to consider the weight changes in relation to the histologic changes in the altered pituitary. The values serve as a standard of comparison for studies on compensatory adrenal hypertrophy in progress at present. Body growth is a fallacious measurement, as the subcutaneous tissues are increased in thickness in the thyroidectomized animal. Kidney weight was thought to be a fair index of visceral growth, as no spontaneous lesions of the kidneys occur that would alter weight, and these organs are not subject to the vascular changes which would introduce a variable factor in their weight, as are the liver, spleen and lungs.

Methods. White rats were thyroidectomized at 24 to 88 days of age, care being taken to avoid trauma to the parathyroids and to the recurrent laryngeal nerves. The rats were killed 76 to 356 days after operation. An equal number of rats were used as unoperated controls. Adequate thyroidectomy was evidenced by retardation of visceral growth, increased

weight of the pituitary, and characteristic histological changes in the pituitary. After the rats were killed with chloroform, the organs were immediately weighed. Appropriate histological sections were made.

RESULTS. The results, presented in brief before the American Physiological Society (9), yielded the following on analysis.

The charts are used instead of tables, as they give concisely all important information, as the number of rats, the extremes of variation, and the mean. Each dot represents the value for one rat.

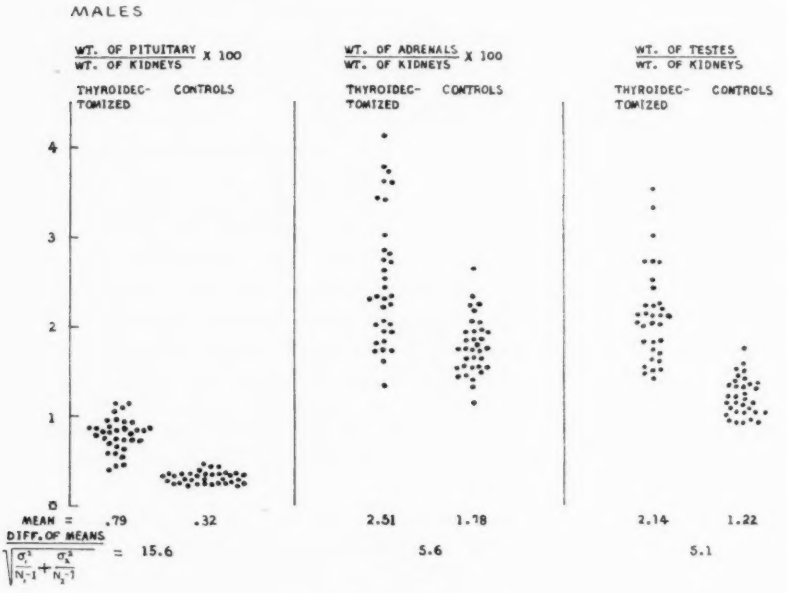

MALES
WT. OF PITUITARY / WT. OF KIDNEYS X 100
WT. OF ADRENALS / WT. OF KIDNEYS X 100
WT. OF TESTES / WT. OF KIDNEYS
THYROIDEC-TOMIZED
CONTROLS
4
3
2
1
0
MEAN = .79 .32 2.51 1.78 2.14 1.22
DIFF. OF MEANS / √(σ₁²/N₁-1 + σ₂²/N₂-1) = 15.6 5.6 5.1

Fig. 1. Ratios in male rats

The ratios of weights of pituitary, adrenals and gonads to kidney weights are shown in figures 1 and 2. The wide spread of the values is due to the great variation in age of the rats; but the values for thyroidectomized and control rats can be legitimately compared, because for each thyroidectomized rat there was a normal rat of the same sex from the same litter or of the same age. That is, all rats were paired, whatever their age. When the difference of arithmetical means divided by the standard error of the difference had a value of two or greater, the difference between thyroidectomized and normal values was regarded as significant.

It will be seen that the ratio of adrenals to kidneys is increased in the

males after thyroidectomy, and that the ratio of testes to kidney is increased. In the female, the ratio of adrenals to kidneys, and the ratio of ovaries to kidneys are not significantly decreased.

Figures 3 and 4 give absolute values instead of ratios. It will be seen that after thyroidectomy there are significant reductions in kidney weights, increase in pituitary weights, decrease in adrenal weights in females, decrease in ovarian weights, but that changes in weights of adrenals in the male and of testes are not significant.

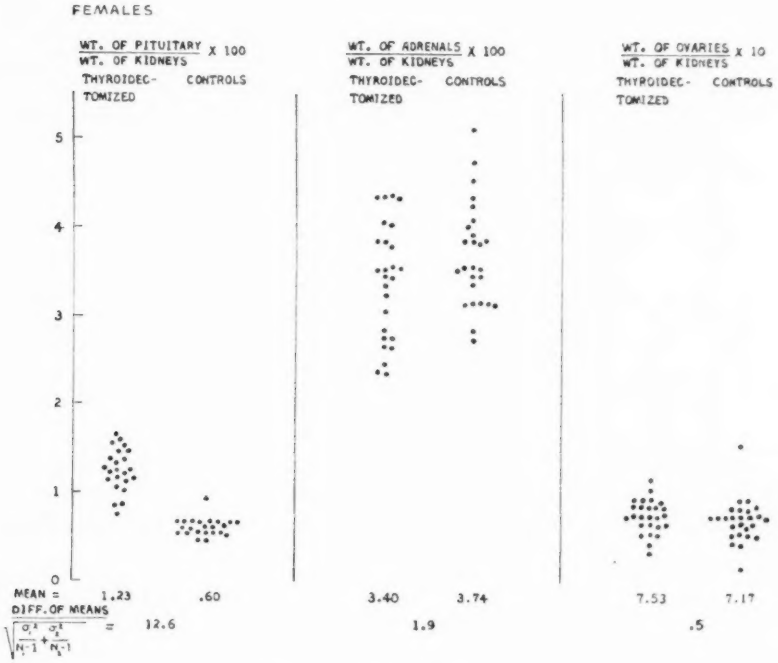


Fig. 2. Ratios in female rats

Histologically the adrenals presented variations in width of cortex but nothing else of significance.

Discussion. There is then in the male a dissociation of adrenal and testis growth from kidney growth. This is particularly pronounced in the case of the testes. Retardation in kidney growth appears to be roughly proportional to degree of loss of acidophiles. Since adrenal growth is maintained in the male thyroidectomized rat in spite of loss of acidophiles, the acidophiles can be excluded as the cells producing the adrenotropic

hormone. As there is no absolute increase in the weight of adrenals or gonads in either sex the large numbers of thyroidectomy cells that crowd the pituitary cannot be secreting an excess of adrenotropic or gonadotropic hormone, unless there are direct effects which prevent the response of these organs to stimulating hormones from the pituitary.

Any reduction in growth of the adrenals in the female might be accounted for by pressure effects in the crowded pituitary upon the cells elaborating adrenotropic hormone, and it will be seen from the data that the female pituitary is enlarged more than the male. The enlargement of

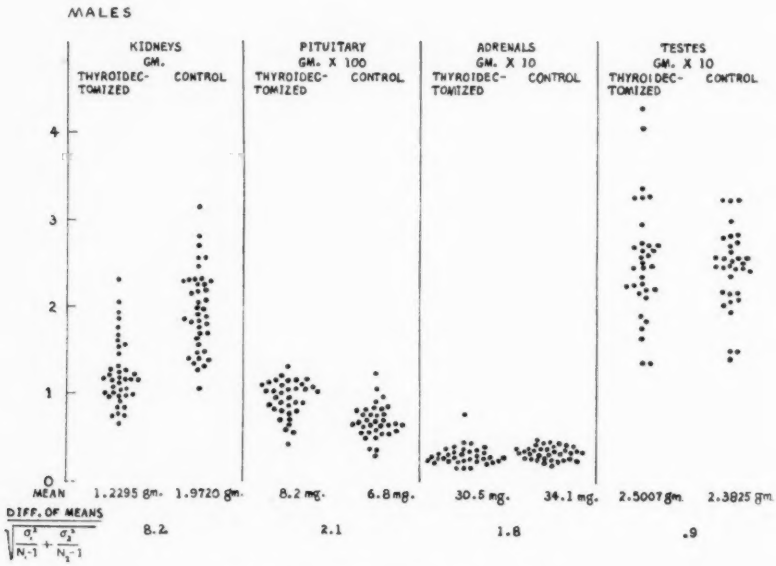


Fig. 3. Absolute weights in male rats

the pituitary is seen grossly to give it a tense rounded congested form, in contrast to the flaccid, flat, pale appearance of control pituitaries.

The only unaltered cells in the pituitary after thyroidectomy are chromophobes and unaltered basophiles. Chromophobes are generally thought to be non-secretory precursors of secretory cells. By a process of exclusion, one may then suggest that cells of a basophile type elaborate the adrenotropic hormone. This is consistent with the findings of Crooke and Russell (1) of secondary changes of vacuolation in some basophiles in patients with Addison's disease. The question then arises whether the same basophilic cell elaborates both gonadotropic and adrenotropic hormones, or

elaborates a common activator substance for both adrenals and gonads, the effect depending upon the end organ, or whether two hormones are elaborated by two types of cells which have not yet been distinguished as separate cells by histological means. From the charts it is seen that adrenal and gonad growth seem to be associated, that is, in the thyroidectomized male rat absolute weights of adrenals and gonads showed no significant changes, but the ratio of adrenal to kidney and of testes to kidney were both increased; while in the female absolute weights were reduced in the case of both adrenals and ovaries. However, when rats are castrated and basophilic castration cells accumulate in the pituitary, such pituitaries elaborate and store an increased amount of gonadotropic hormone and yet no increase of adrenal growth occurs in the female.

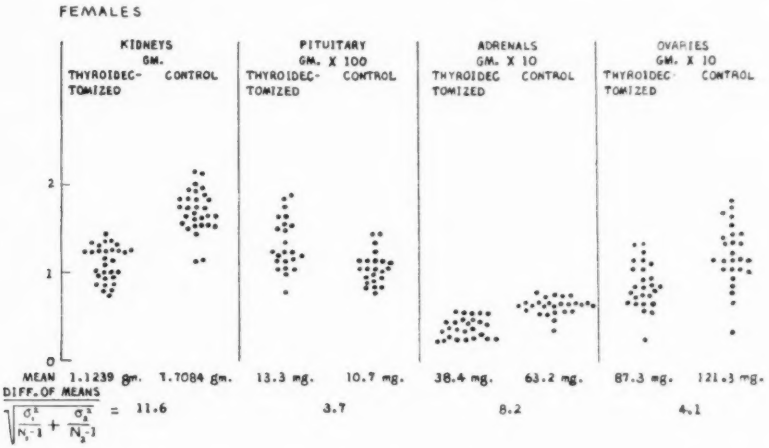


Fig. 4. Absolute weights in female rats

It would seem then that adrenal growth may depend upon a cell that is different in function at least from the basophiles that elaborate gonadotropic hormone.

SUMMARY

1. As a result of thyroidectomy in young rats, there is retardation in kidney growth and increased weight of the pituitary in both sexes.

2. In males there is no significant change in absolute weights of adrenals and testes, but the ratios of adrenals to kidneys, and the ratios of testes to kidneys are significantly increased.

3. In females there is a decrease in absolute weights of the adrenals and gonads but this is proportional to retardation in kidney growth, so that the

ratios of adrenals to kidneys and ovaries to kidneys are not significantly altered.

4. Acidophiles and thyroidectomy cells can possibly be excluded as elaborators of adrenotropic hormone.

5. By a process of exclusion, it is concluded that adrenal growth may depend upon a basophile cell.

REFERENCES

(1) Crooke, A. C. and D. S. Russell. J. Path. and Bact. **40:** 255, 1935.
(2) Evans, H. M. and M. E. Simpson. Anat. Record **45:** 215, 1930.
(3) Hammett, F. S. Endocrinology **10:** 237, 1926.
(4) Hohlweg, W. and K. Junkmann. Pflüger's Arch. **232:** 148, 1933.
(5) Smith, P. E. and E. T. Engle. Anat. Record **45:** 278, 1930.
(6) van Horn, W. M. Endocrinology **17:** 152, 1933.
(7) Zeckwer, I. T., L. W. Davison, T. B. Keller and C. S. Livingood, II. Am. J. Med. Sci. **190:** 145, 1935.
(8) Zeckwer, I. T. This Journal **117:** 518, 1936.
(9) Zeckwer, I. T. Proc. Am. Physiol. Soc., This Journal **116:** 166, 1936.
(10) Zeckwer, I. T. Am. J. Path., 1937.

A STUDY OF PROTEIN ANABOLISM AND CATABOLISM ON A NITROGEN-FREE DIET

WALTER H. SEEGERS[1]

From The Samuel S. Fels Research Institute, Antioch College, Yellow Springs, Ohio

Received for publication July 28, 1937

From our general knowledge of protein metabolism it seems probable that the amino acid requirements for maintenance are quite different from the requirements for growth, but specific information is not plentiful, presumably because experimental difficulties prevent a satisfactory approach to the problem. Actually protein isomerism is involved, and by a conservative estimate (10) the possibilities give figures of astronomical proportions. If, as Fixsen (8) points out, one measured the requirements for growth and maintenance in one experiment and the requirements for maintenance in another, and found the net difference, it would not be possible to rule out a supplementary action of food protein in the experiment in which both functions are operative. If protein anabolism could be superimposed upon a maintenance unit receiving a "N-free" diet no supplementary effect, in the above sense, would interfere, and information could be obtained concerning protein anabolism.

In earlier work it was shown (15) that pregnant rats given a N-free diet as early as 13 days before term give birth to living young. In this condition there exists an anabolic phase of metabolism of considerable magnitude, on a N-free diet. Except for the added factor of the catabolism of newly formed fetal tissue,[2] it can be measured indirectly as metabolism in excess of maternal maintenance requirements. The ma-

[1] Published with the permission of Dr. L. W. Sontag, director of the Fels Research Institute.

[2] We can estimate the importance of this factor as to order of magnitude only. Energy metabolism declines with increasing age (7). The ratio of endogenous urinary nitrogen excretion to basal metabolism is higher for mature than for weanlings (4); but disregarding this and applying the prediction equation (adult animals) given by Brody, Procter, and Ashworth (6), and using the figures for the growth rate of rat fetuses given by Stotsenberg (as cited by Needham (13), p. 1671) the amount of N catabolized by the young can be estimated. Computing for the period of pregnancy considered in table 1 the following is obtained: $N = 146 \times 0.000326^{0.72}$. Hence, approximately 0.438 mgm. more N per day was excreted by each fetus in the second period than in the first period considered in table 1.

ternal reserves[3] must supply the N rather quickly for the growing young and somewhat more slowly for the maintenance of the adult animal itself. If it is assumed that the amino acids are liberated from the maternal reserves in the same manner and through the same mechanism by which they are supplied for maintenance, no wastage should occur in the urine provided the young use them for anabolism in the same proportion in which they are liberated. If, on the contrary, the young select certain of them and discard others the N should appear as wastage in the urine for the same reason that the N of a deficient protein is discarded. The extent of urine N wastage, if any, should be suggestive of the net difference between growth and maintenance requirements.

The above considerations prompted the beginning of the work herein described. Factors given special consideration are: *a*, the combined effect of wastage due to transfer of amino acids from the maternal reserves to the young and that due to the catabolism of newly formed fetal tissue; *b*, the quantity of N which is excreted in the urine after rapid depletion of part of the reserve protein; *c*, the quantity of reserve protein, and *d*, fecal N excretion.

EXPERIMENTAL PROCEDURE. The rats used were select mature breeders obtained from the Sprague-Dawley Company, Madison, Wisconsin. One group of 6 animals was taken from the stock diet on the 11th day of pregnancy, sacrificed, and the young and adnexa analyzed for total N. A second group (experimental or Y series) contained 7 animals, and was taken from the stock diet on the 11th day of pregnancy and given a N-free diet *ad libitum*. The diet was of the same composition as the one used by Seegers and Mattill (16). A vitamin B complex supplement which was fed daily contained 4.3 mgm. N. Urine N collections were made every 2 days for the first 20 days and subsequently every 5 days until the animals succumbed. In the collections and analyses for total N and total creatinine the technique of Ashworth and Brody (5) was used.

Two pooled fecal collections covered the first 20 days and the remainder of the days on the diet respectively. When the animals died the hair was removed by plucking, which was aided where necessary by the application of hot water. The kidneys and liver were then removed, weighed, and analyzed for total N. The remainder of the body was analyzed for

[3] The term "deposit protein" could perhaps be used to good advantage in this connection, but since it apparently will be impossible to make a precise differentiation between "deposit," "floating," "circulating" or some such variety of protein the term reserve protein is simply used. In this paper it represents the quantity of protein an animal, in ordinary nutritive condition, may have available for the maintenance of life when given a N-free diet. This usage unfortunately tends to imply that there is a certain definite supply of protein which is exhausted at death. Death may not be specifically due to such an exhaustion but rather due to an intervening syndrome which is only sooner or later fatal.

total N after it had been hydrolyzed with 25 per cent H_2SO_4. A third group (8 control or C series animals) was placed on the N-free diet for 8 days and urine was collected and analyzed every 2 days. They were then sacrificed, and the kidneys, liver, and the carcasses were weighed and analyzed as described above.

Transfer of nitrogen from maternal reserves. In order to measure the effect of wastage representing amino acids liberated from the maternal reserves and not used by the fetuses and that due to the catabolism of newly formed tissue a suitable basis for comparison is needed. For that purpose it is advantageous to begin feeding the N-free diet on the 11th

TABLE 1

*Comparison of the total N excretion of pregnant and non-pregnant rats fed on a N-free diet**

C-SERIES			Y-SERIES		
Rat number	1st period	2nd period	Rat number	1st period	2nd period
C-1	34.02 (15.95)†	32.26 (15.46)	Y-1	58.09 (23.14)	61.08 (24.28)
C-2	41.13 (19.04)	39.32 (18.67)	Y-2	51.81 (23.71)	60.62 (27.12)
C-3	44.89 (20.49)	43.14 (20.44)	Y-3	55.53 (23.58)	56.10 (23.72)
C-4	38.58 (16.51)	37.76 (16.59)	Y-4	63.11 (24.04)	64.65 (24.12)
C-5	39.17 (19.17)	50.13 (21.45)	Y-6	†	
C-6	41.30 (19.51)	38.42 (18.59)	Y-7	50.24 (22.23)	50.70 (22.43)
C-7	41.39 (18.15)	37.15 (16.56)	Y-8	72.21 (30.99)	81.48 (23.72)
C-8	41.86 (16.54)	33.76 (13.70)	Y-10	47.09 (20.35)	55.00 (26.51)
Average difference. .		−1.30			4.51

* The C-Series animals were control females and the Y-Series pregnant rats. The first period represents the 5th and 6th days on the diet and the second period the 7th and 8th days on the diet.

† As the result of an error, a collection period was improperly spaced. Rat Y-10 is accordingly substituted. The figures in parentheses represent the nitrogen excretion per 100 grams body weight.

day of pregnancy: *a*, because the birth of living young will ensue; *b*, 4 days can be allowed for adjustment to the diet, and *c*, a 2-day collection period (5th and 6th days on diet) can be compared with an adjacent 2-day collection period before the animals begin to refuse an adequate food intake. After parturition the mothers' appetites appear to be good again until a few days before death. The above collection periods are also unique in that there is very little fetal growth in the first 2 days (5th and 6th days on diet) and rapid growth the next 2 days (Stotsenberg as cited by Needham (13, pp. 1671 and 373). Thus a maximum differential is utilized.

In table 1 the figures for urine nitrogen excretion are given for the 3rd and 4th 2 days on the diet, and in chart 1 a graphical representation of all the urine N excretion data obtained on the Y series can be seen. In the control animals the excretion, with one exception, was higher in the first period than in the second. In conformity with previous experiments (15) the reverse was found to be true in the experimental series, and the algebraic difference between the two amounts to 5.8 mgm. daily.

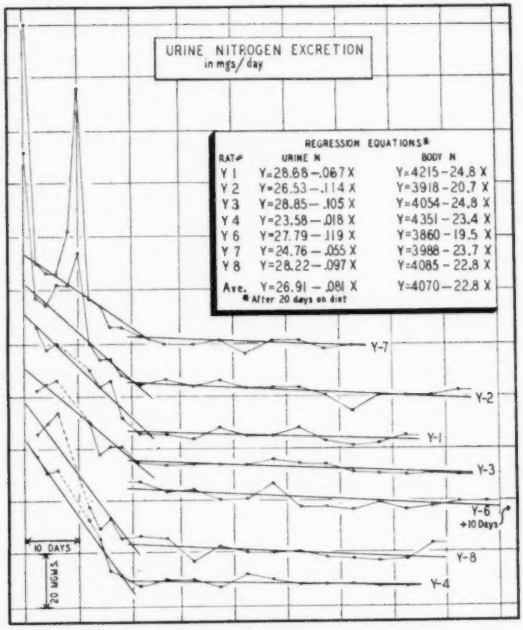


Chart 1. The relative course of urine N excretion is shown completely for rats Y-7 and Y-2. These are typical, and for practical reasons, the first collection periods and the 2 adjacent to birth, are omitted. In the case of Y-6 an error was made in spacing the first collection periods. The regression equations for body N apply to the final period only.

In view of the metabolic processes involved, and since it represents the combined effect of wastage due to selectivity in anabolism and the catabolism of newly formed fetal tissue, this difference is indeed small.[4] There remains, therefore, the possibility that the transfer of the amino acids from the maternal reserves to the fetuses is accomplished without wastage. If there is any wastage it is small in quantity.

In line with the above comparison it can be seen from chart 1 that the

[4] See footnote above.

N excretion on the 4th 2 days is always above the regression line drawn through points representing figures obtained during the first 25 days on the diet. The first 2 days and the 4 days adjacent to the delivery date have for obvious reasons deliberately been deleted. While there may be some question concerning the feasibility of drawing conclusions from this regression line the tendency in urine excretion is clearly visualized.

In all instances the experimental animals excreted a greater quantity of N than the controls. The difference is considerable even if the body

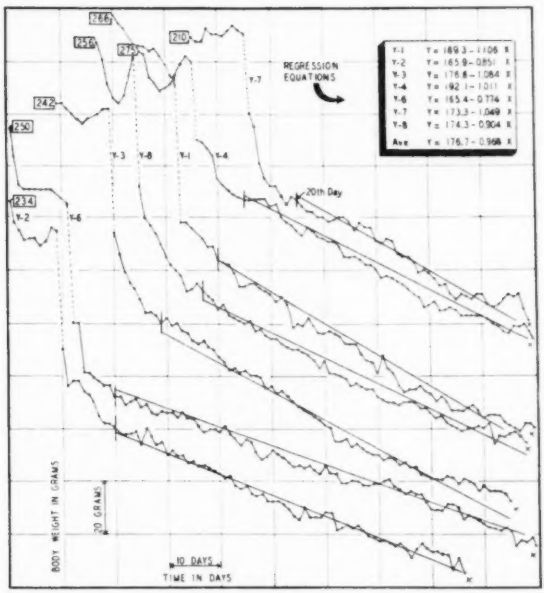


Chart 2. The change in body weight is shown from the start of the experiment on the 11th day of pregnancy until death. Regression equations and lines begin with the 20th day on the diet. The body weight at the beginning is indicated on each curve.

weight, which does not tend to drop in the experimental animals (chart 2), is not first adjusted before the urine N coefficient is computed. This high coefficient is probably caused by the heightened metabolic rate which accompanies pregnancy. Whatever the cause, the figures show that, quite apart from the building of fetal tissue and its maintenance, there is an increase in the protein maintenance requirement during pregnancy.

Urine nitrogen excretion. The course of urine nitrogen excretion on a N-free diet is a matter of considerable importance in biological value

studies where the rat is used. Animals of different species vary in their capacity for adjustment to the endogenous level (18). According to Mitchell (11), and Mitchell and Carman (12) adjustment takes place rapidly in the rat, and the manner in which the ratio urine N/body weight is used in Mitchell's balance sheet method implies that this value should be constant once the adjustment has been achieved. In the work of Ashworth and Brody (5) a question is raised in this connection. They suggest that there may be no absolute minimum level of N excretion. In the work herein described a large quantity of reserve protein was sacrificed to the young and any subsequent adjustment should have given minimum values for urine N.

On chart 1 it can be seen that the urine N excretion curve has two characteristic slopes through which regression lines have been drawn,

TABLE 2

Interrelationship computed from the average of regression equations on charts 1, 2

	TIME ON DIET				
	20 days	40 days	60 days	80 days	100 days†
Body weight (grams)	157.3	137.9	118.6	99.2	79.9
Nitrogen in body (mgm.)	3613	3156	2699	2242	1785
Nitrogen in urine (mgm.)	25.29	23.67	22.05	20.43	18.81
$\frac{\text{Urine N (mgm.)}}{\text{Body weight (grams)}} \times 100$	16.07	17.15	18.59	20.58	23.54
$\frac{\text{Body N (mgm.)}}{\text{Body weight (mgm.)}} \times 100$	2.296*	2.287	2.275	2.258	2.234

* In these figures the weight of the skeleton becomes of more influence with time on the diet.

† Not all animals on the diet lived for 100 days.

the transition point from one to another being between the 20th and 25th days on the diet. We may, at this point, disregard the first 20 days and consider the remainder of the time on the diet. In these animals the excretion gives excellent correlations with the straight line equation $Y = a + bX$. The excretion approaches constancy in the absolute sense, and not in terms of the ratio urine N/body weight (actually there was a decline of 0.081 ± 0.01 mgm. per day, which is not much, but is of some importance when animals are kept on such a diet over a long period of time). This ratio increases (see table 2) because the body weight declines at the rate of 0.96 ± 0.03 gram per day (chart 2) while the urine nitrogen excretion declines very little. Ashworth (4) does not emphasize this point but gives 2 typical charts which show an increase in this ratio after the initial decline. The variability in this ratio is, therefore, apparently also found in normal animals.

The metabolic interrelationships on a N-free diet change in such a manner that the per cent nitrogen content of the body does not change. Heretofore this point has not been stressed. Since the entire animal (without hair, which is regarded as having no metabolic function) was analyzed at death, as were the feces and urine as described above, the necessary data are available for computing the N content of the animal while it was on the diet. The regression equations for the decline in body weight are given on chart 2, and the per cent composition of the body, as computed from group average regression equation constants, is given in table 2. Taking cognizance of the influence of skeletal weight, the composition of the soft tissue is seen to remain constant. On a relatively normal and constant food intake which in accordance with the work of Schneider (14) would avoid variation in fecal N, one could predict the urine N loss from the rate of decline in body weight. With the data on charts 1 and 2 a fairly good prediction figure for total nitrogenous loss can be obtained. Body weight declined 0.968 ± 0.03 gram per day (coefficient of variation = 12.11 per cent) and the total N excretion amounted to 22.8 ± 4.77 mgm. per day (coefficient of variation = 8.2 per cent). Hence 23.4 mgm. N were lost per gram of body weight loss. We may reason further that a loss of 0.968 gram in body weight and 22.8 mgm. of N yields figures for obtaining the per cent N in the soft tissue of the body, or $\frac{22.8}{968} \times 100 = 2.34$ per cent N in the body. This compares satisfactorily with the figure in table 2, and is proof that the hair is properly regarded as dead tissue.

As a supplement to these figures one can compute the ratio of $\frac{a}{b}$ from the constants in the regression equation ($Y = a + bX$) for decline in body weight and for the decline in body N (charts 1 and 2) and obtain a statistical abstraction representing the number of days which must elapse before all body weight or all body N would be exhausted. When this computation is done for body weight the figures are $\frac{176.7}{0.968} = 186.5$ days; for body nitrogen they are $\frac{4070}{22.85} = 178.1$ days. The writer regards the difference as experimental error. This conclusion is more clearly indicated when individual scores are computed. This fact is additional evidence in favor of the conclusion that the per cent of N in the soft tissue remains approximately constant, and that the figures in table 2 would indicate this fact more precisely if the weight of the skeleton were ruled out.

Quantity of reserve protein. The work of Addis, Poo and Lew (1) (2) (3) has shown that the quantity of N lost by different organs varies considerably, and that there is no uniformity in the absolute or relative anabolism or catabolism of various body parts. In terms of quantity

the organs showing trends of special interest in the aforecited work can contribute relatively little reserve nitrogen in comparison with the total quantity of available reserve protein.

Much protein can be liberated quickly. This fact is shown by the composition of the young and placentas (table 3) at birth which, in these experiments took place in cages with large glass mesh bottoms so that the young and/or placentas would not be eaten. This rapid liberation of N was accompanied by an increase in the excretion of total creatinine, which was determined on each Y series animal for the first 20 days on the diet. The figures for rat Y-1 are typical and are as follows

TABLE 3

*The quantity of nitrogen (expressed in milligrams) lost in the young and placentas at birth**

RAT NUMBER	NUMBER OF YOUNG	NITROGEN IN YOUNG	NITROGEN IN YOUNG AND PLACENTAS†
Y-1	10	529	552
Y-2	10	529	536
Y-3	10	431	431
Y-4	13	644	685
Y-6	3	160	181
Y-7	5	242	242
Y-8	9	470	546
Y-10	10		

* The quantity lost can, for practical purposes, be regarded as a total loss to the maternal reserves; because at the time the animals were started on the diet (11th day of pregnancy) the young contained very little N. Six rats were sacrificed on the 11th day of pregnancy. The fetuses were fixed in dilute acid after the uterine horns had been severed on either side. After fixation the uterine wall was pealed away and discarded. The remainder of the material contained on the average 1.63 mgm. N. On the 11th day the trophoblast is penetrated by the umbilical vessels and the first interchange of food substance between maternal and fetal blood systems occurs (13, p. 1671).

† The placentas were frequently eaten despite the wide mesh bottom of the cage. On the average they contained 7.64 mgm. N.

(milligrams creatinine excreted per day): 11.11, 9.90, 14.39, 18.99, 17.61, 12.19, 7.54, 7.18, 6.80, and 7.40. This behavior is different from that of non-pregnant rats (5), and at least in humans (17) there is no consequential change in total creatinine excretion with time in pregnancy. This rapidly liberated protein is undoubtedly from reserves otherwise available for maintenance since the survival period of all these animals was less than that of a non-pregnant animal (beginning weight 176 grams) which lived 126 days on the same diet before she died.

As much as 64.4 per cent of the normal protein supply (table 4) of the animal can be used before it succumbs, and the loss in body weight

is of the same order of magnitude. With one exception the absolute as well as the per cent nitrogen content of the kidneys and the liver was lower in the experimental animals at death than in the controls which had themselves been on the diet 8 days. In the case of the liver the

TABLE 4

Composition of liver, kidneys, and entire body at death

		EXPERIMENTAL (Y-SERIES)			CONTROL (C-SERIES)		
		Weight	N content		Weight	N content	
		grams	*per cent*	*mgm.*	*grams*	*per cent*	*mgm.*
Liver	M.....	4 17 ± 0 06	1 92 ± 0 03	80 19 ± 3 35	9 35 ± 0 26	1 93 ± 0 04	180 62 ± 5 74
	V†.....	5 23	0 68	16 42	11 65	8 43	13 33
Kidneys	M	0 98 ± 0 03	2 00 ± 0 06	19 44 ± 0 48	1 51 ± 0 04	2 33 ± 0 01	35 31 ± 0 92
	V.....	13 68	10 92	9 65	11 55	2 29	10 94
Body	M.....	101	2 19 ± 0 00*	2228 9	217 1 ± 0 02	2 24 ± 0 02	4882 6
	V.....		0 846			3 36	

* The influence of skeletal weight has not been factored out. If this were done the nitrogen content of the entire soft tissue would be shown to be much higher than that of the liver and kidneys.

† The coefficient of variation. All figures for the control series are based on 8 animals and those for the experimental series on 7. The controls were killed after they had been on the diet 8 days.

TABLE 5

The per cent loss in body nitrogen and body weight on N-free diet

RAT NUMBER	BODY N*	BODY WEIGHT
Y-1	59.29	59.77
Y-2	58.66	59.83
Y-3	60.00	62.80
Y-4	54.58	57.09
Y-6	64.37	63.45
Y-7	52.85	51.67
Y-8	59.20	58.98
Average...	58.42	59.08

* The quantity consumed in the food has been factored out.

TABLE 6

Fecal nitrogen excretion on N-free diet

RAT NUMBER	FECAL N PER DAY		FOOD INTAKE PER DAY		FECAL N / URINE N
	1st 20 days	Final period	1st 20 days	Final period	
	mgm.	*mgm.*	*grams*	*grams*	*per cent*
Y-1	11.41	7.43	5.90	6.89	29.25
Y-2	10.85	6.61	6.95	6.35	32.25
Y-3	9.72	7.20	7.15	5.77	30.91
Y-4	12.19	8.57	6.35	6.04	37.09
Y-6	8.19	7.03	5.80	5.52	33.65
Y-7		9.29	6.50	5.96	41.49
Y-8	12.77	6.86	*	5.53	31.23
Average					33.69

* An error was made which vitiates the record for a 6-day period.

difference was nil; but the prolonged drain diminished the absolute content, and from the small coefficient of variation in per cent composition it seems likely that an absolute lower limit was reached. No other coefficient of variation in table 4 is so low. Only the coefficient for the N

content of the body in the experimental series can in any way compare with it.

Fecal nitrogen excretion. Work on fecal nitrogen excretion has mostly been done for the purpose of improving methods for determining biological values. In the present work the feces excreted the first 20 days constituted one pool and the subsequent period another. Table 6 contains a comparison of the 2 periods. In all cases less N was excreted in the second period, and the difference cannot be explained on the basis of food consumption. In the first period a 24-hour intestinal rate was assumed, and probably an error of 24 hours was thereby incurred. If, however, we discard the first 6 days in which the effect of the previous diet might be a disturbing factor, and compare the per day excretion of the 6 to 20th day period with the final period the figures for the latter are smaller. It seems logical to suppose that a lessened digestive waste should be a part of the body's adjustment in favor of nitrogen economy.

The figures for the ratio fecal N/urine N are also given in table 6. Brody, Procter and Ashworth (6) have computed this ratio from the data of Fixsen and Jackson (9), and for those of Terroine and Reichert (19). The values found were 20.4 per cent and 60 per cent respectively, while for their own mature animals they found 27.29 $\pm$ 0.63 per cent. Allowing for the variation in the figures in table 6 the average of 33.6 per cent cannot be regarded as being much higher than that of Brody, Procter and Ashworth, but differs considerably from that of the other authors (9) (19). It is easy to see how the difference between 27.29 and 33.69 could result from different levels of food ingestion or from slight differences in collection periods and consequent differences in stages of adjustment in urine and/or fecal N excretion.

SUMMARY

When rats are given a nitrogen-free diet during pregnancy the N for the young is transferred to them from the maternal reserves. If there is any wastage of N in this transfer, as the result of the young selecting a different assortment of amino acids than is liberated from the maternal reserves, it should appear in the urine. The newly formed tissue of the young will also have a catabolic phase of metabolism yielding N. The combined effect of wastage due to transfer of amino acids and that due to the catabolism of the young was measured and found to be small.

Pregnant animals have a much higher urine N coefficient than non-pregnant control animals, which suggests a higher metabolic rate. After the animals had given birth to young and had thus used a large quantity of their reserve protein they were continued on the diet until they died. During the final period (after the first 20 days on the diet) the total urine N excretion approached constancy. A decline of 0.081 $\pm$ 0.01 mgm.

per day was observed. The ratio urine N/body weight increased while the body weight and total N content of the body declined at a rate which gave a constant for the per cent N in the body.

Although the food intake was approximately the same during the first 20 days as during the final period, N excretion was found to be less in the latter period. The ratio fecal N/urine N was found to be 33.69 per cent.

At death the animals had lost on the average 59.08 per cent of their original body weight and 58.42 per cent of their original N supply. The weight and N content of the liver and kidneys was found and compared with animals which were on a N-free diet for only 8 days.

REFERENCES

(1) Addis, T., L. J. Poo and W. Lew. J. Biol. Chem. **116:** 343, 1936.
(2) Addis, T., L. J. Poo and W. Lew. J. Biol. Chem. **115:** 111, 1936.
(3) Addis, T., L. J. Poo and W. Lew. J. Biol. Chem. **115:** 117, 1936.
(4) Ashworth, U. S. Missouri Agric. Exper. Res. Bull. no. 223.
(5) Ashworth, U. S. and S. Brody. Missouri Agric. Exper. Sta. Res. Bull. no. 189.
(6) Brody, S., R. C. Procter and U. S. Ashworth. Missouri Agric. Exper. Sta. Res. Bull. no. 220.
(7) Cooperative Enterprise. Missouri Agric. Exper. Sta. Res. Bull. no. 176.
(8) Fixen, M. A. B. Nutrition Abst. and Rev. **4:** 447, 1935.
(9) Fixen, M. A. B. and C. M. Jackson. Biochem. J. **26:** 1919, 1932.
(10) Leaths, J. B. Science **64:** 387, 1926.
(11) Mitchell, H. H. J. Biol. Chem. **58:** 873, 1924.
(12) Mitchell, H. H. and G. G. Carman. J. Biol. Chem. **68:** 183, 1926.
(13) Needham, J. Chemical embryology. The Macmillan Company, New York, 1931.
(14) Schneider, B. H. J. Biol. Chem. **109:** 249, 1935.
(15) Seegers, W. H. This Journal **119:** 474, 1937.
(16) Seegers, W. H. and H. A. Mattill. J. Biol. Chem. **110:** 531, 1935.
(17) Seegers, W. H. and M. Potgieter. Human Biol. **9:** in press.
(18) Smuts, D. B. J. Nutrition **9:** 403, 1935.
(19) Terroine, E. F. and T. Reichert. Arch. Intern. Physiol. **32:** 337, 1930.

STAGES OF DEVELOPMENT OF RESPIRATORY REGULATION AND THE CHANGES OCCURRING AT BIRTH[1]

MORRIS ROSENFELD AND FRANKLIN F. SNYDER

From The Department of Pharmacology and Experimental Therapeutics and the Department of Obstetrics, The Johns Hopkins University

Received for publication July 28, 1937

The present investigation is concerned with the regulation of respiration in the newborn. It is prompted by recent findings regarding the influence of carbon dioxide and oxygen upon the respiratory movements of fetuses within the uterus (Snyder and Rosenfeld, 1937). In contrast to the adult animal, the fetus is relatively insensitive to an excess of carbon dioxide although a certain tension of carbon dioxide is required for maintenance of respiration; and in response to anoxemia the fetus is depressed by a degree of oxygen want which stimulates respiration in the adult. The present study represents an attempt to trace the transition between the fetal type of response and that of the adult animal. It involves the determination of whether the adult type of respiratory regulation develops gradually during the early days of postnatal life or whether there is an abrupt change at the time of delivery. A second question was also investigated, namely, at what stage of development does sensitivity to excess carbon dioxide and low oxygen first appear in newborn animals delivered prematurely.

METHOD AND MATERIAL. Respiratory tracings were obtained in newborn rabbits by means of a small plethysmograph connected with a recording tambour (fig. 1). Amplitude was recorded (table 1) in terms of the excursion of the lever measured in centimeters. No anesthesia was used. The head of the animal rested in an outer chamber through which gas mixtures of known composition circulated. Temperature was maintained at a constant level throughout an experiment.

In order to detect any changes which occur abruptly at birth, the animals were speedily placed in the plethysmograph following delivery so that the first tests were applied within 15 to 30 minutes after birth. The stage of pregnancy was accurately known since the rabbits were mated in the laboratory.

Observations are based upon 22 newborn rabbits obtained from 11

[1] Presented before the American Physiological Society at Memphis, Tennessee, April 21–24, 1937. (Proceedings published in This Journal **119:** 406, 1937.)

litters at various stages of development ranging from the 28th to 32nd day of pregnancy and including three animals observed 10 days following spontaneous birth at full term.

OBSERVATIONS. *Anoxemia.* The present observations were begun in rabbits ten days of age. Respiratory tracings showed clearly that stimulation followed the administration of a low oxygen mixture (4 per cent oxygen in nitrogen), an increase occurring in both the rate and amplitude of respiratory excursions. This response of the 10 day old rabbit was in striking contrast to the depression of respiratory movements which was

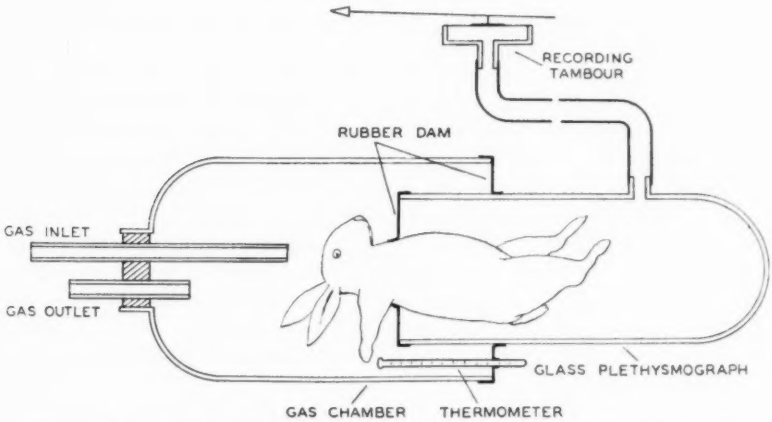


Fig. 1. Plethysmograph for recording the respiratory movements of the newborn; gas chamber of glass to permit administration of various gas mixtures. Dimensions of plethysmograph, 4 cm. diameter x 12 cm.

regularly observed in fetuses within the uterus when subjected to anoxemia by administration of a low oxygen mixture to the mother (Snyder and Rosenfeld, 1937).

Newborn rabbits were next examined. It was known from previous experiments that with anoxemia, apnea of the fetus and hyperpnea of the mother occurred not only in fetuses at term but also in postmature fetuses retained within the uterus at 35 days, i.e., 3 days past term. Therefore, in normal newborn rabbits at 32 days a crucial test was afforded as to whether or not the change in response to anoxemia was related to birth.

In the newborn at 32 days in which respiratory tracings were taken within a few hours following spontaneous delivery, low oxygen markedly stimulated respiration. Additional evidence of the onset of the adult

type of response from the moment of birth was obtained in 31 day rabbits which were delivered by hysterotomy and in which records of respiration were obtained at once. Stimulation by anoxemia was well marked. It was evident that the change from depression within the uterus to stimulation after delivery occurred at the time of birth instead of developing gradually during early postnatal life. Depth of breathing was markedly increased, while the rate was much less influenced.

In order to determine at how early a stage of development the respiratory system was first stimulated by low oxygen, premature animals were delivered by hysterotomy at 29, 28 and 27 days. Respiratory tracings obtained at 29 days revealed definite stimulation in response to low oxygen. Depth of breathing increased more than 50 per cent while the rate remained essentially unchanged. At 28 days low oxygen repeatedly failed to stimulate either rate or amplitude of respiratory movements. No immediate depression occurred such as was noted in fetuses within the uterus. At 27 days, several litters were delivered but the animals succumbed within a half hour, although kept at a temperature of 37°. Reliable respiratory tracings were not obtained. The animals delivered at 28 days survived usually for several hours but were obviously immature and soon died. The 29 day animals were viable and remained alive in the laboratory for several days without special care. It is significant to note that stimulation of respiration by low oxygen occurs throughout the stage of viability and its absence is first observed in animals incapable of survival.

Nitrogen. The results obtained with administration of pure nitrogen confirmed the findings with the 4 per cent oxygen mixture. Stimulation was obtained with nitrogen and was followed within about a minute by depression or paralysis of respiratory activity. In the experiments with 4 per cent oxygen paralysis of respiration was not reached in the five minute experimental period usually adopted. In two of the three fetuses tested with nitrogen at 28 days there was no initial phase of stimulation, but depression immediately set in.

The observations with nitrogen were based upon 15 experiments, using 10 animals representing all of the stages of development which were included in the study of carbon dioxide and oxygen.

Cyanide. To apply a further test in addition to anoxemia as to whether or not the carotid body mechanism was functional immediately after delivery, the response to cyanide was investigated. Sodium cyanide administered intravenously in unanaesthetized animals immediately after delivery resulted in a sudden sharp response. Stimulation was immediate and was marked by a striking increase in depth as well as rate. Regarding the time of origin of the response, it was found that in animals delivered at the beginning of the stage of viability, i.e., 29 days, stimulation was

already clear cut. In four animals delivered at 28 days, stimulation was obtained in two of them. Sodium cyanide was given in a dosage of

TABLE 1

Respiratory response of the newborn to carbon dioxide excess and oxygen deficit at various stages of development

STAGE OF PREGNANCY	NEWBORN NUMBER	LITTER NUMBER	CARBON DIOXIDE 7½ PER CENT				OXYGEN 4 PER CENT			
			Rate		Amplitude		Rate		Amplitude	
			Before	After	Before	After	Before	After	Before	After
days										
28	1	166	52	68	1 2	1 5	104	102	1 1	1 3
			60	64	1 2	1 2	116	116	1 1	1 4
	2	28	76	80	0 5	0 5	70	70	0 5	0 5
			76	80	0 4	0 6				
	3	27	18	20	0 5	0 5				
			22	21	0 6	0 6				
	4	27	32	34	0 6	0 6	52	48	0 6	0 6
			52	52	0 6	0 6	54	54	0 6	0 6
29	5	19	80	104	1 8	2 3	132	104	1 7	3 0
			116	116	1 5	2 3	132	124	1 7	3 0
	6	103	96	96	1 9	2 8	104	96	1 4	2 2
			100	116	1 9	2 7	96	96	1 2	1 8
	7	104	104	128	1 7	3 0	108	108	2 0	2 4
			108	128	2 0	3 1	122	112	1 4	2 0
	8	103					96	96	1 0	1 9
							104	108	1 1	2 2
31	9	106	104	104	1 1	2 0	114	108	1 4	1 6
			132	124	1 2	2 2	132	136	1 1	1 5
32	10	105	112	112	1 9	3 8	108	116	2 0	3 3
			120	112	1 7	3 7	120	108	2 0	2 9
	11	17	84	104	1 2	3 2	104	126	2 0	4 0
			112	120	2 0	6 7				
10 days post-partum	12	125	30	38	0 5	0 9	40	80	0 7	1 9
			34	40	0 7	0 9	40	72	0 5	2 0
	13	125	48	44	1 9	3 5	38	132	1 6	7 5
			40	42	1 5	3 0	54	112	1 6	4 0

The measurements of rate and amplitude of respiratory movements were made from plethysmograph records obtained from newborn rabbits. Respiratory rate per minute is given and the amplitude of the excursion of the recording lever is expressed in centimeters. Two representative experiments are tabulated for each animal although additional tests were obtained.

0.05 cc. of a M/350 to M/1000 solution. In the animals studied at 28 days, stimulation followed injection of 0.05 cc. of M/1000 sodium cyanide,

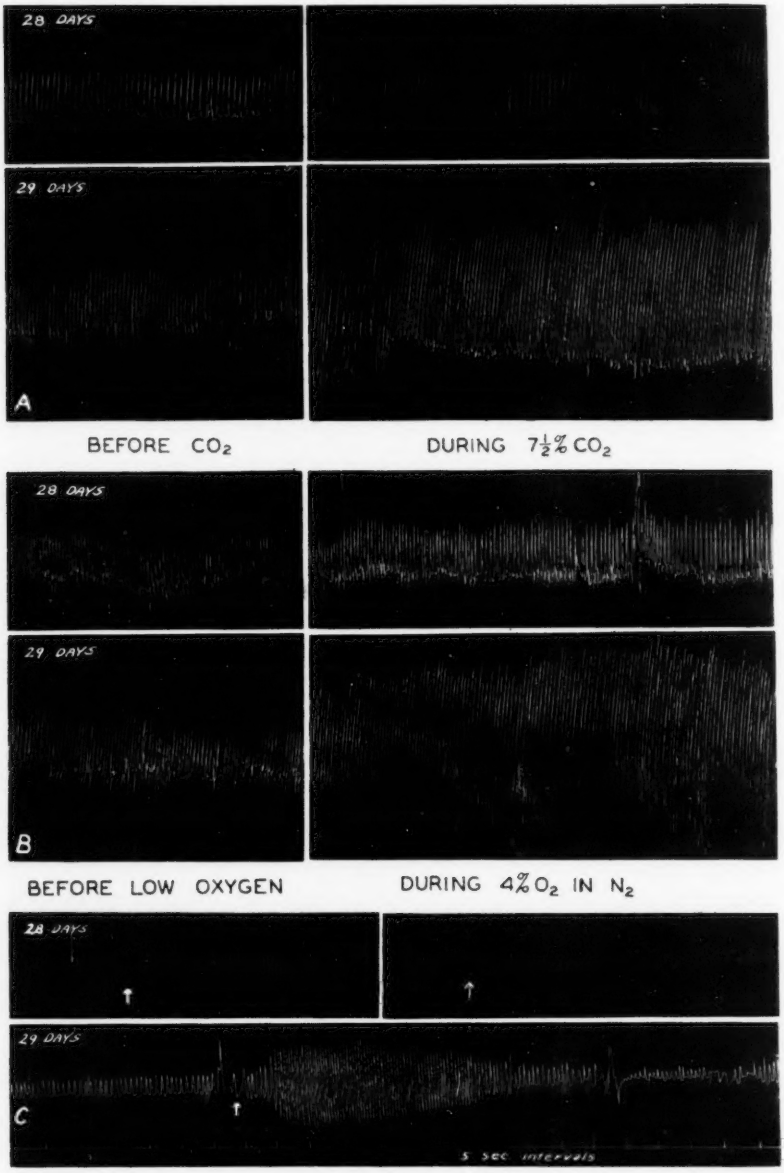

28 DAYS
29 DAYS
A
BEFORE CO_2
DURING $7\frac{1}{2}\%$ CO_2
28 DAYS
29 DAYS
B
BEFORE LOW OXYGEN
DURING 4% O_2 IN N_2
28 DAYS
29 DAYS
C
5 sec intervals
RESPONSE TO NaCN

Fig. 2

while the same amount of a more concentrated solution, namely, 0.05 cc. of M/350 resulted in depression. The animals ranged in weight from 30 grams at 28 days to 45 grams at 32 days. Control injections of physiological salt solution gave no response. Observations upon the response to sodium cyanide are based upon 30 tests in 11 newborn rabbits which include 4 animals at 28 days, 3 at 29 days, 2 at 32 days, and 2 animals 10 days after spontaneous birth at term.

Excess carbon dioxide. The response of the newborn to excess carbon dioxide was investigated in an attempt to determine any changes occurring at birth. The influence of carbon dioxide was observed in the same series of animals in which the effect of low oxygen was studied. Carbon dioxide was administered at the beginning of an experiment before low oxygen was tried, in order to eliminate any possible injury of the respiratory center resulting from anoxemia.

In animals born spontaneously at term and studied at various ages ranging from 10 days to a few hours, clearcut stimulation followed administration of 7½ per cent carbon dioxide. Depth of breathing increased more than 50 per cent while the rate showed little change. Thus, at birth a striking sensitivity to excess carbon dioxide was noted which was not demonstrated in the fetus within the uterus.

The stage of development at which the newborn first showed sensitivity to excess carbon dioxide was investigated in animals removed from the uterus at 31, 29 and 28 days. Respiratory tracings obtained at 31 and 29 days showed definite stimulation. Depth of breathing increased more than 75 per cent at 31 days and at least 50 per cent at 29 days, while the rate remained essentially unchanged. At 28 days no response to excess carbon dioxide could be demonstrated. It is evident that sensitivity to excess carbon dioxide is present in animals born throughout the period of prematurity, and first appears at the same stage as anoxemic stimulation, namely, at the beginning of viability.

High oxygen. The attempt was made to observe any response to a change from air to pure oxygen in the course of the experiments. We had in mind two possible types of response to be investigated: first, a

Fig. 2. Respiratory tracings showing the stage of development of the respiratory system at which sensitivity first appears to stimulation by excess carbon dioxide, low oxygen and cyanide. Records obtained from premature rabbits placed in the plethysmograph immediately following delivery by hysterotomy. A. At 28 days, no stimulation by excess carbon dioxide. At 29 days, stimulation is first elicited. B. At 28 days, no stimulation by low oxygen. At 29 days, stimulation is first elicited. It is interesting to note that birth results in reversal of the response to anoxemia, depression occurring within the uterus in contrast to stimulation immediately following delivery. C. At 28 days, slight stimulation by sodium cyanide 0.05 cc. M/1000 (first arrow). Depression by large dose, 0.05 cc. M/350 (second arrow). At 29 days, stimulation by sodium cyanide 0.05 cc. M/350.

stimulation due to improved oxygenation of the respiratory center; secondly, a depression resulting from the removal of an anoxemic stimulus already present (Marshall and Rosenfeld, 1936). Neither type of response was observed at any stage of development. Respiration remained unchanged upon the administration of oxygen.

Discussion. The regulation of breathing immediately after birth becomes a matter of special interest in view of the striking contrast between the mechanism of respiratory control observed in the fetus within the uterus and that of the adult animal. It is remarkable that in the intrauterine environment the fetus is relatively insensitive to the most powerful stimulants of adult respiration, namely, excess carbon dioxide and low oxygen. Anoxemia, indeed, was purely depressant. It was known from the previous study that this type of response persisted in postmature fetuses retained within the uterus 3 days past term as a result of inhibition of parturition. This observation suggested that transition to the adult type of response occurred at a later stage of development. A surprising finding of the present experiments is that the change is abrupt, appearing immediately after delivery. In fact, the change is related to birth rather than to the stage of development. The full term animal delivered at 32 days responds like the adult while the fetus, as long as it remains within the uterus, even at 35 days, continues to respond like fetuses of earlier stages.

In addition to parturition itself, a second critical period in the transition from the fetal to the adult type of response was detected at the beginning of the stage of viability, at which time there first appeared the capacity to respond with stimulation to carbon dioxide excess, oxygen want and cyanide. The acquisition of a highly sensitive regulatory mechanism for the control of respiration parallels closely the capacity of the animal to survive outside of the uterus. The respiratory movements which occur throughout a large part of embryonic life are of functional significance in the dilatation of the air passages before birth (Snyder and Rosenfeld, 1937), but fail to provide adequate respiratory exchange after delivery in the absence of a regulatory mechanism. It is evident that control of respiratory movements by development of sensitivity to carbon dioxide excess and to oxygen deficit is an essential adaptation for independent existence outside the maternal body.

It was previously pointed out that the respiratory activity of the fetus in the intrauterine environment closely resembled that of an adult animal after denervation of the carotid body area. The present observations upon the newborn throw considerable light upon the question as to whether the failure of functional activity of the carotid body mechanism of the fetus is related to structural undifferentiation or whether it is due to inhibition of activity of an organ capable of functioning. Stimulation

in response to anoxemia and also to cyanide in the newborn delivered at various stages throughout the period of viability revealed that the carotid body is capable of functioning long before term. The abrupt reversal of the effect of anoxemia in the brief interval occupied by escape of the fetus from the uterus eliminates incomplete structural development as a factor and hence leads to the conclusion that before birth there is inhibition of function of the carotid body mechanism.

In brief, the finding of a peculiar type of respiratory regulation in the fetus presented a new problem, namely, the question of the time of origin of the adult type of response. By examination of a series of normal newborn and premature animals, two critical points in the transition between intrauterine and postnatal respiratory regulation emerge clearly: 1, the time of birth when an abrupt change occurs to the adult type of response; 2, the beginning of the period of viability when the capacity of the respiratory system to respond to excess carbon dioxide, and low oxygen first becomes differentiated, although inhibited from functioning within the uterus.

SUMMARY

1. Stimulation of respiration in the newborn in response to excess carbon dioxide, anoxemia and cyanide first appeared at the beginning of the stage of viability, namely, at 29 days.

2. Before the period of viability, regular rhythmical respiration occurred, although the absence of stimulation following excess carbon dioxide, low oxygen or cyanide demonstrated the immature development of the regulatory mechanism.

3. The transition from the intra-uterine to the adult type of respiratory response occurred abruptly at birth instead of developing gradually during postnatal life.

4. The failure of the carotid body mechanism to function in the intrauterine environment is not due to structural undifferentiation, since characteristic responses to anoxemia and cyanide are present immediately after birth.

REFERENCES

MARSHALL, E. K., JR. AND M. ROSENFELD. J. Pharmacol. and Exper. Therap **57**: 437, 1936.

SNYDER, F. F. AND M. ROSENFELD. This Journal **119**: 153, 1937.
Proc. Soc. Exper. Biol. and Med. **36**: 45, 1937.

FURTHER OBSERVATIONS ON THE POTENTIAL DISTRIBUTION IN THE FROG'S GASTROCNEMIUS MUSCLE DURING ACTIVITY[1]

C. A. MAASKE, M. R. KRASNO AND J. A. E. EYSTER

From the Physiological Laboratory, University of Wisconsin, Madison

Received for publication July 28, 1937

In a preceding communication (1) it has been shown that the potential changes occurring during the activity of the gastrocnemius muscle of the frog stimulated through its motor nerve, are adequately described by the occurrence, within the muscle, of two coaxial asynchronous electrical dipoles. The orientation and period of growth and decay of these dipoles were determined by an analysis of potential time curves recorded from different regions in a circular conducting field surrounding the muscle. In the experiments described in the present communication this conclusion has been confirmed by a different method of analysis and the approximate length and position of the dipoles determined.

METHOD. If the potential is measured at various points along a line parallel to and close to the axis of a dipole, maximum positive and negative potentials are found at points opposite or nearly opposite the positive and negative poles of the dipole. At a point opposite the mid point of the dipole axis, the potential is zero. If the potentials are measured at successive points beginning proximal to one pole and ending distal to the other and plotted, a diphasic curve results. The distance between the positive and negative peaks of this curve approximates closely the length of the dipole or the distance between the positive and negative charges.

Wilson, Macleod and Barker have made theoretical analyses of the effects due to *a*, a single dipole; *b*, a number of dipoles lying along parallel axes, and *c*, a number of dipoles radiating from a common point. The mathematical functions plot as diphasic curves. The distance between the maximum and minimum values of the functions approximate closely the length of the dipole when the distance of the line along which the potentials are measured from the dipole axis is small. As this distance is increased, the distance between the maximum and minimal values becomes longer than the length of the dipole axis.

We have carried out a number of physical experiments with a dipole formed from the two poles of a battery at the center of a circular conducting field and have found that the situation described above holds, within the

[1] Supported in part by a grant from the Wisconsin Alumni Research Foundation.

limits of measurement, even when the line along which the potentials are measured is further from the dipole axis than can possibly be present when the potentials along a muscle are measured by surface contacts.

The method that we have employed consists, then, in measuring the maximum potentials at a number of surface contacts along the long axis of the muscle and determining the points of maximum positive and negative values. For this purpose a multicontact electrode was used (fig. 1) by means of which the potentials could be recorded from 18 to 20 points along the long axis of the muscle. The upper surface of a bakelite block has a series of small openings 1.6 mm. apart, each of which communicates with a single one of the upright glass tubes. The isolated gastrocnemius muscle

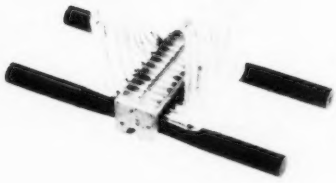


Fig. 1

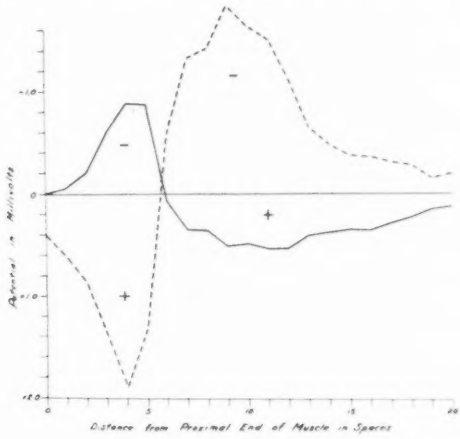


Fig. 2

Fig. 1. Multicontact electrode for recording potentials at successive contacts along the surface of the muscle.

Fig. 2. Maximum potentials plotted for 20 points along the surface of a frog's gastrocnemius muscle.

was fastened to the block in such a way that nearly isometric contractions resulted when the muscle was stimulated and each electrode opening was in direct contact with its surface. The preparation was immersed in Ringer's solution in a circular dish. The ground electrode leading to a two stage direct coupled amplifier was placed at the margin of the dish at a point along an axis passing through the middle of the muscle and at right angles to its long axis. Our previous experiments have shown that the potential along this axis is zero throughout the contraction of the muscle. The electrode leading to the grid of the amplifier was placed in one of the upright tubes, making contact with the muscle through the Ringer's solution with which it was filled. Both electrodes were of the zinc-zinc sulphate

type. The amplifier led to a string galvanometer, by means of which the potentials were recorded. The muscle was stimulated through its nerve, contact with the stimulating electrode being made well above the surface of the bath. Under these conditions and when using a low voltage (0.4 to 0.6 volt) condenser discharge for stimulation, no detectable stimulus escape occurs.

Starting at one end of the muscle, the action potentials were recorded from the surface at each of the successive points. Usually the records were checked by repeating the procedure from the opposite end of the muscle.

Results. The data from a typical experiment are given in figure 2. The maximum potentials are shown in millivolts for 20 points along the muscle surface. The left end of the abscissa scale corresponds to the femoral end of the muscle. The solid line shows the maximum potentials at each point due to the first dipole. This dipole has its negative charge approximately 6 mm. from the femoral end of the muscle. The position of the positive charge is difficult in this case to locate accurately, but it is approximately 10 mm. distant from the negative charge along the long axis of the muscle. The maximum potentials due to the second dipole are shown by the broken curve. Its positive charge corresponds in position with the negative charge of the first dipole. The negative charge of the second dipole is approximately 8 mm. from the positive charge along the long axis of the muscle toward its tendon end. The points of zero potentials, or approximate mid point of the two dipole axes, nearly coincide.

The type of distribution described above occurred in all but a few of the experiments performed, and we regard it as representative of the normal. Occasionally the points of zero potential occur nearer the tendon end of the muscle and still less frequently there are two points of zero potential for each curve. In these cases the potentials beyond the second point are small.

SUMMARY AND CONCLUSIONS

The fact that the potentials developed in the gastrocnemius muscle of the frog stimulated through its motor nerve can be adequately described by two asynchronous dipoles directed along the long axis of the muscle, previously deduced from an analysis of potential time curves from a conducting field surrounding the muscle, is confirmed in the present experiments in which a different method of approach to the problem was used. The present method was to measure maximum potentials developed at contacts along the surface of the muscle. When these are plotted against distance along the muscle it is found that the two dipoles are approximately 8 mm. in length and have their proximal poles about 6 mm. from the femoral end of the muscle.

REFERENCES

(1) Eyster, J. A. E., F. Maresh and M. R. Krasno. This Journal **111:** 641. 1935.
(2) Wilson, F. N., A. G. MacLeod and P. S. Barker. University of Michigan Studies, Scientific Series, Vol. 10. 1933. Also J. Gen. Physiol. **16:** 423. 1933.

STUDIES ON THE PANCREAS AND LIVER OF NORMAL AND OF ZINC-FED CATS

D. A. SCOTT AND A. M. FISHER

From the Connaught Laboratories, University of Toronto, Toronto, Canada

Received for publication July 29, 1937

During the past few years the relation between zinc and insulin has interested us greatly in these Laboratories. It was shown (Scott, 1934) that many commercial preparations of insulin contained zinc, and that this metal was a constituent of insulin crystals prepared by the methods then available. When this small quantity of zinc was removed from the insulin, crystallization could no longer be effected unless zinc, nickel, cobalt or cadmium was added at some stage of the process. Based on these observations, a practical method for the preparation of large quantities of insulin crystals was described. Essentially this method differed from previous ones in that it involved the addition of small amounts of one or more of the metals. It was found that the metal-content of crystals of insulin was constant for each metal and was uniformly proportional to the atomic weight of the metal used in the process of crystallization (Scott and Fisher, 1935). In biological experiments it was shown that solutions of insulin containing added zinc produced a more retarded hypoglycemic effect in rabbits and mice than did similar solutions containing no added zinc (Scott and Fisher, 1935). More recently we have demonstrated that the presence of minute quantities of zinc in suspensions of protamine and insulin not only greatly increases the stability of these preparations but also prolongs their blood-sugar lowering effect (Scott and Fisher, 1936).

Although zinc is present in all tissues and organs of the body (Itallie, 1907), and in amounts almost as great as that of iron (Lutz, 1926), its physiological function has not as yet been clearly demonstrated. It has been shown by Bertrand and Bhattacherjee (1934) and by Stern, Elvehjem and Hart (1935), that zinc is a necessary constituent of the diet for the normal growth of mice and of rats. Thompson, Marsh and Drinker (1927) fed large amounts of zinc to albino rats. They observed that such animals produced offspring which grew normally during their early life. In similar experiments these authors (Drinker, Thompson and Marsh, 1927) showed that dogs and cats could be maintained on a high zinc diet without impairing the health of the animals. On this regimen the concentration of zinc was increased in the liver, gall bladder and bile, gastro-intestinal tract and kidneys—that is, in the excretory organs. In three cats receiv-

ing large doses of zinc over a long period of time, fibrotic changes in the pancreas were observed. Otherwise these authors report that histological examination of the organs of such dogs and cats showed no damage attributable to zinc.

Because of the close chemical relationship between insulin and zinc, and because of the fibrotic changes in the pancreas noted by Drinker et al. in certain cats receiving zinc, it seemed possible that this metal might be a factor in the liberation, utilization or storage of insulin. Accordingly the experiments reported herein were undertaken to determine if there were any significant relations between the insulin content and zinc content of the pancreatic glands of cats maintained on a basal diet or on a diet containing added zinc. Since the liver also is concerned with carbohydrate metabolism, it was decided to estimate the zinc in this organ as well as in the pancreas.

EXPERIMENTAL. Thirty healthy male cats were selected. They were divided into two groups of fifteen each. The average weight per cat in each group was approximately 3500 grams. Each cat was placed in a cage approximately 2 feet by 2 feet by 1 foot and the cages numbered.

The diet of the cats consisted mainly of minced ox heart or canned salmon. On five days of each week, each cat was fed 100 grams of the heart per day, and on the other two days 100 grams of fish per day. Each cat received approximately 75 cc. of milk per day. In addition one group of cats (nos. 16–30) received a weighed amount of highly purified zinc oxide daily. This compound was thoroughly mixed with the meat. At the beginning of the experiment each of these cats was given 0.50 gram of zinc oxide per day. However, it was found that certain cats either did not eat their food containing the metal or vomited the greater part of their meal. Accordingly the amount of zinc oxide given to each cat per day was decreased to 0.25 gram. The cats ate their food normally for about one month. An attempt was then made to increase the amount of zinc oxide to 0.40 gram per day, but again difficulties in eating were encountered, and after a few days the amount of metal was decreased to 0.30 gram per day. This amount of the compound was added daily to the diet until the completion of the experiment, a period of from three to four months. During the last few weeks of the experiment it was observed that certain cats receiving the zinc did not eat all their food. This was particularly noticeable in cats 18, 19, 26 and 28. Moreover, the general condition of this group of cats was not as good as was that of the cats in the control group (nos. 1–15).

At the end of three months, one or two cats were killed each day. Such cats were deprived of all food for sixteen hours. They were then weighed. Each cat was stunned by a sharp blow on the head and the carotid artery immediately severed. A sample of blood was collected and sugar estima-

tions were made in duplicate. The sugar was determined by the modified Shaffer-Hartman method (DeLong, 1927) and the values are recorded for each cat in tables 1 and 2. After a cat had bled out an autopsy was immediately performed and the pancreas and liver were removed. These organs were each weighed and minced with stainless steel scissors. Zinc estimations were made on minced portions of the pancreas and of the liver. Insulin estimations were made on a portion of the pancreas.

Insulin estimations. Three grams of pancreas were weighed on a watch glass and finely minced by means of scissors. (In certain cats receiving the zinc diet the pancreas was small and consequently a smaller amount of pancreas and extraction fluid was used for the estimations.) This material was then transferred to a 50 cc. centrifuge thimble and to it were added 12 cc. of an acid alcoholic solution. (Several litres of this extraction fluid were prepared and portions of it used for extracting the pancreas of each cat. It consisted of 750 cc. absolute alcohol, 15 cc. concentrated hydrochloric acid, and 250 cc. water.) After standing over night the mixture was filtered through a double layer of cheese-cloth and pressed until nearly dry. The solid material was again minced and re-extracted with 12 cc. of the acid alcohol for two hours. The alcoholic extract was again filtered through cheese cloth and the two extracts combined. This filtrate was made ammoniacal and the volume measured. The mixture was filtered through filter paper and two 9.0 cc. quantities pipetted into each of two 50 cc. centrifuge thimbles. To each tube were now added 15 cc. of absolute alcohol and 25 cc. of ether. The mixtures were shaken and placed in the refrigerator over night. They were then centrifuged, the supernatant alcohol-ether discarded, and the tubes allowed to drain for one hour. The insulin in each tube was then dissolved in 25 cc. of isotonic saline (pH 2.5). The potency of these solutions was then estimated by the mouse method of assay (Scott, 1936). Generally six tests were conducted involving at least 200 mice. From the average of these potency values the number of units of insulin per gram of pancreas was calculated. The results for each pancreas are recorded in tables 1 and 2.

Zinc estimations. All samples of pancreas and of liver for zinc estimations were digested with 5N HCl. A quantity of this acid was prepared by distilling 20 per cent hydrochloric acid (CP) in pyrex glassware, and diluting the distillate to the desired strength with distilled water. A spectroscopic analysis of this solution gave a negative test for zinc.

In the control group a 10 gram sample of liver and a 3 gram sample of pancreas were used for the estimation of the zinc in these organs of each cat. These samples were cut into small pieces by means of stainless steel scissors and put into 100 cc. pyrex digestion flasks. To the sample of liver and of pancreas were added 50 cc. and 15 cc. respectively of the acid digestion liquid. These mixtures were refluxed for 1 hour and were then allowed to

stand over night. The following morning the volume was measured and each mixture filtered through acid washed filter paper. The zinc content of aliquots of each solution was estimated spectroscopically and the amount of zinc per gram of tissue calculated. These results are shown in table 1.

In the group of cats maintained on the zinc diet a similar procedure was followed. It was necessary, however, to use smaller samples of glands or to dilute the digestion mixture after hydrolysis, on account of the high zinc content. When the pancreas was small, the former procedure was used and a 1 gram sample was digested with 15 cc. of the 5N HCl solution.

TABLE 1

The insulin and zinc content of cat pancreas, and the zinc content of the liver. Basal diet

CAT NUMBER	INITIAL WEIGHT	FINAL WEIGHT	BLOOD SUGAR	LIVER WEIGHT	PANCREAS WEIGHT	Zn PER GRAM PANCREAS	TOTAL Zn PER PANCREAS	Zn PER GRAM LIVER	TOTAL Zn PER LIVER	INSULIN PER GRAM PANCREAS	TOTAL INSULIN PER PANCREAS
	grams	*grams*	*per cent*	*grams*	*grams*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*units*	*units*
1	3,830	3,925	0.110	75	7.00	0.077	0.53	0.053	3.98	1.91	13.37
2	3,355	3,475	0.100	80	6.45	0.065	0.42	0.058	4.68	1.46	9.42
3	3,825	3,860	0.103	76	9.00	0.050	0.45	0.041	3.14	0.80	7.20
4	3,005	3,320	0.107	60	8.75	0.073	0.64	0.066	3.96	1.34	11.73
5	3,135	3,810	0.141	78	6.50	0.085	0.55	0.050	3.91	2.22	14.41
6	3,500	3,610	0.117	91	8.20	0.032	0.26	0.100	9.12	1.63	13.36
7	3,815				4.70					1.90	9.00
8	4,380	4,670	0.095	118	7.45	0.071	0.53	0.059	7.02	1.24	9.24
9	3,470	3,800	0.103	76	7.10	0.084	0.60	0.034	2.69	2.31	16.40
10	3,030	3,520	0.102	77	9.95	0.111	1.10			0.80	7.96
11	4,100	4,470	0.107	59	4.83	0.087	0.42	0.050	2.96	2.90	14.01
12	2,500	3,600	0.099	81	8.12	0.072	0.59	0.041	3.35	1.56	12.67
13	3,430	3,310	0.092	73	6.00	0.092	0.55	0.036	2.63	2.01	12.06
14	2,710	3,500	0.099	73	5.52	0.046	0.25	0.089	6.46	1.50	8.29
15	3,800	3,400	0.094	64	8.20	0.061	0.50	0.071	4.53	1.67	13.69
Average	3,459	3,734	0.105	77.2	7.18	0.072	0.53	0.058	4.49	1.68	11.52

Cat 7 developed a severe infection and was killed thirty days after commencing the experiment.

In other cases, a 3 gram sample was used and after hydrolysis an aliquot was diluted with the 5N HCl. In estimating the zinc in the livers of this group of cats sometimes 3.3 gram samples were digested with 50 cc. of the 5N HCl, in other cases 10 gram samples were digested with 50 cc. of the 5N HCl and then further diluted with the digestion liquid. The amount of zinc in the various digests was estimated spectroscopically as in the normal group of cats and the results are recorded in table 2.

The spectroscopic analyses were conducted by Dr. S. Bateson of the Department of Physics of this University. Details of the method, limit of error ± 3 per cent, will be published elsewhere.

Pathological findings. All the cats used in this study, cat 29 excepted, were autopsied immediately after death. Sections from the heart, thyroid, lymph gland, spleen, pancreas, liver, kidney, adrenal, duodenum and terminal ileum were examined. The samples were fixed in formol saline, embedded in paraffin and stained with hematoxylin and eosin.

In the case of all the cats receiving the zinc diet, there was evidence of various degrees of damage to the pancreatic glands. Degenerative changes in the acinar tissue seemed to occur first. These were followed by an influx of lymphocytes and reticulo-endothelial cells, and later fibroblasts.

TABLE 2

The insulin and zinc content of cat pancreas, and the zinc content of the liver. Zinc diet

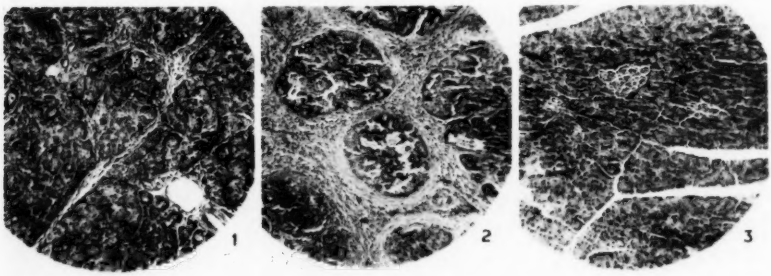
CAT NUMBER	INITIAL WEIGHT	FINAL WEIGHT	BLOOD SUGAR	LIVER WEIGHT	PANCREAS WEIGHT	Zn PER GRAM PANCREAS	TOTAL Zn PER PANCREAS	Zn PER GRAM LIVER	TOTAL Zn PER LIVER	INSULIN PER GRAM PANCREAS	TOTAL INSULIN PER PANCREAS
	grams	*grams*	*per cent*	*grams*	*grams*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*units*	*units*
16	2,850	2,540	0.100	62	2.60			1.22	76.10	2.57	6.68
17	3,495	2,480	0.126	61	2.95	0.39	1.14	0.94	57.34	2.90	8.55
18	3,275	Died		92	1.80	0.70	1.25	1.00	91.49		
19	3,615	2,675	0.110	96	5.50	0.52	2.86	1.04	100.32	1.61	8.86
20	3,625	2,460	0.112	72	6.20	0.49	3.05	0.88	63.36	1.63	10.11
21	3,660	3,100	0.097	104	6.20	0.44	2.70	0.59	61.88	2.83	17.55
22	3,325	2,970	0.105	61	5.20	0.56	2.93	0.96	58.38	1.62	8.43
23	5,355	4,855	0.094	133	6.30	0.54	3.40	0.77	102.81	3.63	22.87
24	3,530	2,500	0.111	60	2.12	0.41	0.87	1.27	75.90	6.49	13.75
25	3,465	2,000	0.189	72	7.05	0.44	3.08	1.19	85.82	1.11	7.83
26	2,660	2,050	0.107	47	3.45	0.44	1.51	0.61	28.84	2.31	7.97
27	4,110	2,635	0.147	62	2.62	0.44	1.15	1.00	61.68	2.31	6.05
28	4,320	3,080	0.098	69	3.85	0.30	1.14	0.35	26.15	2.93	11.28
29	3,100	Died		101	3.70	0.61	2.26	1.14	115.14		
30	3,625	2,510	0.191	107	3.70	0.44	1.63	0.52	55.64	1.40	5.18
Average	3,600	2,758	0.121	79.9	4.22	0.48	2.07	0.90	70.72	2.56	10.40

Cats 18 and 29 died 91 and 90 days respectively after the zinc-feeding commenced. All cats were autopsied with the exception of no. 29. Marked fibrotic changes in the pancreas of all cats were observed.

The end result commonly seen in this group of cats consisted of small nodular pancreatic glands which gross examination showed to be of a gritty nature. In the microscopic sections one could see bands of fibrous tissue separating or replacing areas of acinar tissue and resulting in a lobulated appearance. These changes are evident in figures 1 and 2, which are photomicrographs of glands of cats 23 and 30 (zinc diet). The control group receiving no zinc revealed none of these changes and all the pancreatic glands examined were normal in appearance (fig. 3). No differences in the islet tissue of the pancreas were observed between the cats on the basal diet and those on the high zinc diet.

In addition, however, to the striking changes in the pancreas, it should be noted that the spleen of many cats receiving the zinc diet often showed collections of degenerated cells and small amounts of amyloid deposits. In two animals in this group the adrenals were found to be hard and areas resembling calcification (or zincification?) occurred in the cortex.

The livers and kidneys of both groups appeared to have undergone varying degrees of intoxication as evidenced by failure to stain properly. Vacuolization, and loss of cell substance, was also apparent, particularly in the kidneys. Frozen sections stained with scharlach-R showed that a large amount of fat was present. Since such changes occurred in both groups one cannot say at this time that the zinc-feeding was specifically concerned. No changes deemed significant in this experiment were found in the other tissues examined.



Figs. 1 and 2 are photomicrographs of sections of the pancreases of cats 23 and 30. Both these cats were maintained on a high zinc diet for approximately 15 weeks, eating their food regularly. Bands of fibrous tissue separating or replacing areas of acinar tissue and resulting in a lobulated appearance are in evidence.

Fig. 3. A photomicrograph of a section of the pancreas of a cat (no. 11) maintained on the basal diet.

Discussion. The object of the present experiment was to determine the relation, if any, between the ingestion of large amounts of zinc and the storage of insulin and of the metal in certain organs. Such conclusions as can be drawn are made as a result of observations with two groups of fifteen cats each, one group being maintained on a basal diet, and the other group on a similar diet to which zinc oxide was added.

From the average values recorded in tables 1 and 2 it can be seen that the ingestion of large quantities of zinc caused no significant decrease in the total insulin content of the pancreas of the cats. Although the average quantity of insulin per pancreas was 10 per cent less in those cats on the zinc diet than in those of the control group, this difference is not much greater than the error in the biological assay of insulin content of the various solutions. It is probably of greater importance, however, to note

that although the average *total* amount of insulin per pancreas did not vary greatly from one group to the other, there was considerable variation (45 per cent) in the average amount of insulin per *gram* of pancreas. From a consideration of these facts and in view of the comparatively small size of the pancreatic glands of the cats maintained on the zinc diet, we are inclined to believe that, while the ingestion of zinc may *appear* to increase the amount of insulin *per gram* of pancreas, the *total* insulin content of the gland remains unaltered for the reasons set forth above.

The changes in the pancreatic glands which were observed at autopsy are interesting. It will be noted that there was a marked difference in the size of the pancreases obtained from the two groups of cats. In addition the pancreatic glands from all the cats which received zinc were of a hard, gritty nature, showing marked fibrotic changes. (Cat 29 was not autopsied.) Microscopic sections showed bands of fibrous tissue separating or replacing areas of acinar tissue (figs. 1 and 2). No difference in the islet tissue of the pancreases was observed between the cats on the basal diet and those receiving added zinc. The changes in the size and the nature of the pancreatic glands of the cats on the high zinc diet can be attributed, in our opinion, only to the ingestion of the zinc oxide. Care was taken to select a grade of such purity (arsenic less than 0.0005 per cent; heavy metals less than 0.01 per cent) that the ingestion of other metals was not significant. There remains the possibility that some of the degenerative phenomena observed in this group could be due to malnutrition since certain cats did not eat regularly during the last month and there was an average decrease of several hundred grams in weight during the experiment. From the results of the control group it is evident that the food, if ingested, was totally adequate to permit maintenance of body weight. Although, in the early stages of the experiment, we made many changes in the diet and the quantity of metal, we were not able to attain the success reported by Drinker, Thompson and Marsh (1927), who reported that all their cats ate heartily, even when ingesting a larger amount of zinc than was used in the present work. These authors, however, observed in their experiments, with 10 cats and 3 dogs on a high zinc diet, that the pancreas of three cats showed marked fibrotic changes.

In the cats on the basal diet the average zinc concentration per gram of liver and of pancreas was 0.058 mgm. and 0.072 mgm. respectively (table 1). The value for liver agrees with that found by Lutz (1926) and by Drinker, Thompson and Marsh (1927). The concentration of zinc in the pancreas, however, is approximately three times that found by these investigators. Our results indicate that the pancreas is a richer organ in zinc than the liver. From table 2 it will be seen that the zinc concentration in the pancreas and the liver was increased in the cats on a high zinc diet 7 and 15 times respectively above their normal content.

In summarizing these experiments the great variation amongst even the control group allows only general conclusions such as have been made. For example, in table 1 it is recorded that there were two cats whose pancreatic glands were approximately the same weight as the average value recorded for the zinc-diet cats. Until a satisfactory explanation for this and similar variations can be offered, conclusions can only be drawn from the average of a large number of experiments.

SUMMARY

Cats fed on a high zinc diet for from 12 to 16 weeks showed a loss in weight averaging approximately 900 grams per cat. The amount of zinc per gram of pancreas and of liver was about 7 and 15 times, respectively, as great as that found in similar organs in the control group of cats. The average weight of the liver in each group of cats was approximately the same. The average weight of the pancreas of the cats receiving the zinc diet was only approximately half that of the control group. The total insulin in the pancreas of each group of cats was approximately the same, although on account of the great difference in size, the insulin content per gram of pancreas was higher in the cats receiving the zinc diet than in the control group. Marked fibrotic changes in the pancreas of all the cats on a high zinc diet were observed.

The authors are indebted to Dr. D. L. MacLean, who conducted the autopsies and prepared the pathological report recorded in this paper.

REFERENCES

Bertrand, G. and R. C. Bhattacherjee. Compt. rend. Acad. **198:** 1823, 1934.
DeLong, W. A. J. Biol. Chem. **72:** 731, 1927.
Drinker, K. R., P. K. Thompson and M. Marsh. This Journal **80:** 31, 1927.
Itallie, V. Ned. Tijdschr. Geneesk. **63:** Pt I, 1709, 1907.
Lutz, R. E. J. Industr. Hyg. **8:** 177, 1926.
Scott, D. A. Biochem. J. **28:** 1592, 1934.
Scott, D. A. and A. M. Fisher. Biochem. J. **29:** 1048, 1935.
J. Pharmacol. and Exper. Therap. **55:** 206, 1935.
J. Pharmacol. and Exper. Therap. **58:** 78, 1936.
Scott, D. A. Quart. Bull. Health Organ., League of Nations **5:** 607, 1936.
Stirn, F. E., C. A. Elvehjem and E. B. Hart. J. Biol. Chem. **109:** 347, 1935.
Thompson, P. K., M. Marsh and K. R. Drinker. This Journal **80:** 65, 1927.

SOME EFFECTS OF ACUTE ANEMIA ON THE TRANSMISSION OF IMPULSES THROUGH A SYMPATHETIC GANGLION

D. BARGETON[1]

From the Department of Physiology in the Harvard Medical School

Received for publication July 30, 1937

While the effects of anemia on the elements of the cerebrospinal centers have been the object of numerous studies, these effects have been much less investigated in the sympathetic ganglia. Langendorff (1891, 1894), Langley (1894, 1901), Hering (1903) and Tuckett (1905) mention incidentally the exceptional resistance to anemia of cells belonging to the autonomic nervous system. Schröder (1907) seems to have been first to study in a sympathetic ganglion the effects of interrupting the circulation. He observed the long-lasting persistence of ganglionic activity after the death of the animal by bleeding and the possibility of restoring activity, once it had disappeared, by perfusion with Ringer's solution introduced through the carotid artery. Cannon and Burket (1913) pointed out the high resistance of the cells of the mesenteric plexus to complete anemia produced by compression of the gut between two glass plates. Eccles (1935), in a study of the electrical response of the superior cervical ganglion, observed a decrease in the size of the electrical complex and a lengthening of the latent period following interruption of the circulation, and remarked that a very minute blood supply is sufficient to maintain functional activity. Working on perfused ganglia, Bronk and Larrabee (1937) observed an increase of the electrical response and a persistent facilitation in a ganglion deprived of its blood supply.

The present investigation has been aimed at a study of some of the effects observed in the functioning of a sympathetic ganglion when its circulation was impaired.

METHOD. Cats were used, under dial anesthesia (Ciba, 0.8 cc. per kilogram, given intraperitoneally).

The superior cervical sympathetic ganglion (s.c.g.) was chosen because it is connected with a reliable and easily recorded indicator, the nictitating membrane (n.m.), and because it has a readily approachable blood supply.

Isotonic contraction of the n.m. was recorded by means of a lever having a magnification of 14 and exerting a tension of 5 grams.

[1] Fellow of the Rockefeller Foundation.

The preganglionic fibers were stimulated by means of silver silver-chloride shielded electrodes applied to the cervical sympathetic nerve. The chain was crushed below the level of stimulation. The electrodes, isolated by cotton pads, were connected through a transformer to a multivibrator furnishing rectangular waves. Supramaximal shocks were used.

Suppression of the blood supply to the s.c.g. was effected by clamping the common carotid after exclusion of all collateral circulation. The main arteries feeding the s.c.g. branch out from the common carotid in the region of the carotid sinus. Collateral vessels run along the trunk of the vagus and the postganglionic fibers; others come less frequently from the wall of the pharynx. When these collateral vessels have been cut, the ganglion is dependent for its vascularization only on the common carotid. If the external and internal carotids have been tied above the s.c.g. and the superior thyroid tied below it, clamping of the common carotid will entirely suppress the blood supply.

Practically, to make sure that all collateral vessels were tied, the whole region of the carotid bifurcation was dissected, leaving the s.c.g. adherent to the carotid sinus, and cutting between two ligatures all the vessels in the region except the common carotid and the internal jugular vein below the ganglion. The nerves emerging from the skull with the postganglionic trunk were severed to allow a careful dissection of the latter. In the first experiments the ganglion nodosum lying on the side of the s.c.g. was removed; later it was found better to cut only the trunk of the vagus and leave the ganglion nodosum adherent to the s.c.g., thus avoiding possible injury. Such dissection respects the integrity of the s.c.g. and allows it to show an entirely normal activity during more than 4 or 5 hours.

On the opposite side the s.c.g., without any dissection, was used as a control. The preganglionic fibers were stimulated and the contraction of the n.m. was recorded as on the side under experiment. The stimulating electrodes of both sides were fed by transformers, the primary circuits of which were mounted in series, thus providing the same stimulation.

When the preganglionic fibers were continuously stimulated the contraction of one n.m. was recorded under normal and the other under modified circulatory conditions in the s.c.g. It is known that under prolonged stimulation fatigue in the ganglionic cells has to be taken into consideration (Orías, 1932) and that signs of fatigue appear only if the frequency of stimulation is above a certain limit, approximately 20 per sec. In a first series of experiments the frequency of stimulation was kept below that rate in order to eliminate intervention of fatigue. In a second series higher frequencies of stimulation were used and the effects of impaired blood supply were observed together with the fatigue manifestations.

Results. *A. Frequency of stimulation less than 20 per sec.* In such

conditions the ganglionic response, manifested by the indicator, keeps a constant level practically indefinitely, as shown by the record of the control side.

Complete suppression of the blood supply. Complete suppression of the blood supply results in a complete loss of ganglionic activity. When the common carotid is clamped there is a first period, lasting 2 to 3 minutes, during which no visible change occurs. Thereafter the response con-

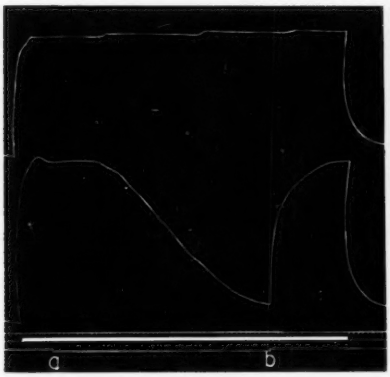


Fig. 1

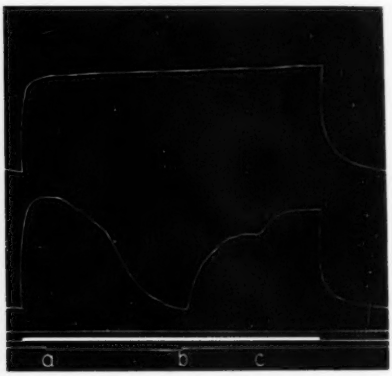


Fig. 2

Fig. 1. Effects of complete suppression of the blood flow through the superior cervical ganglion on the response of the nictitating membrane. The middle signal denotes maximal stimulation of both cervical sympathetics; frequency: 14 per sec. Between *a* and *b* the right common carotid was clamped. In this and the succeeding figures the upper record is that of the control left nictitating membrane; the lower record is that of the right membrane; the time signal records 30 sec. intervals.

Fig. 2. Effects of partial suppression of the blood flow through the superior cervical ganglion on the response of the nictitating membrane. At *a* the right common carotid and the untied lingual artery were clamped, completely shutting off the blood supply to the ganglion. At *b* the clamp on the lingual artery was released, permitting some blood to flow through. At *c* the clamp on the common carotid was removed, reëstablishing a normal circulation. Frequency of stimulation (middle signal): 9 per sec.

tinuously decreases until, after 10 to 15 minutes, the tracing falls to the base line and there remains. If the clamp is removed, functional recovery occurs more or less rapidly and the contraction of the n.m. reaches the same level as before (see fig. 1).

Influence of the repetition and the duration of periods of anemia. When, after apparently complete functional recovery, the s.c.g. is again deprived of blood, the effects of the second period of anemia closely resemble the effects of the first in duration and rate of decline and recovery. After

repeated interruptions, however, slight changes occur—the curves show a more abrupt decline, and a more delayed and slower recovery.

When the blood supply has been interrupted only a short time, the recovery following renewal of the circulation occurs after a brief latent period of a few seconds and progresses rapidly, the curve rising in an almost vertical line. A very short period (5 to 15 sec.) of restored circulation results in a functional recovery lasting several minutes, although meanwhile the circulation has been again interrupted.

When the circulation has been stopped for a long time, the functional recovery appears only after a latent period of several minutes and pro-

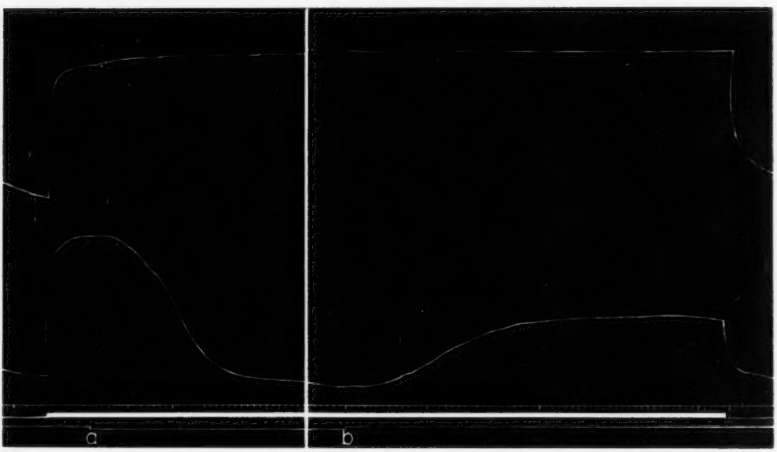


Fig. 3. Complete suppression of the blood supply to a ganglion for 70 minutes (between *a* and *b*) with partial recovery after reëstablishment of the circulation. Fifty minutes of record were cut between the two parts illustrated. Frequency of stimulation: 9 per sec.

gresses slowly. After 48 minutes a very rapid and almost complete recovery has been observed; it has been much slower and only partial after 70 minutes (see fig. 3).

Incomplete suppression of the blood supply. When a small vessel has escaped ligation, such as the small artery running parallel to the vagus or those accompanying the postganglionic bundles, the minute blood supply thus provided may be sufficient to mask partially or entirely the effect of carotid occlusion. The anemia is only made complete by section of the remaining vessel. If, instead of tying the external carotid above the origin of the ganglionic branches, a clamp is put on that vessel and another on the lingual artery which arises about a centimeter from its

beginning, it is easily possible to deliver blood to the ganglionic arteries at different pressures—at normal carotid pressure (clamp on common carotid removed), or at recurrent pressure through the external carotid (clamp on common carotid alone) or at more reduced recurrent pressure through the lingual artery (clamps on common and external carotids, clamp on lingual artery removed). The effects of supplying the s.c.g. with blood at reduced pressure are variable from one animal to another, depending probably on variable vascular arrangements. In some animals no difference is observed, whether the ganglion is supplied at the normal carotid pressure or at reduced pressure through the lingual artery. In others, each level of blood pressure has a corresponding level of response, a higher response attending a higher pressure and remaining relatively constant as long as the pressure is maintained (see fig. 2).

Influence of the frequency of stimulation. The frequency of stimulation, varying from 5 to 20 per sec., does not exert an appreciable influence upon the effect of acute anemia on the s.c.g.; the duration and the time-course of this effect remain the same.

B. Frequency of stimulation higher than 20 per sec. In such circumstances it is known that the ganglionic response after a more or less prolonged peak decreases and that the contraction remains at a relatively constant level or slowly falls (Orías, 1932). Once the constant level has been reached the clamping of the common carotid results first in a *rise* of the record lasting 4 to 5 minutes. This rise is followed by a fall until the record reaches the base line, in about the same time as it does, in the same animal, when lower rates of stimulation are used (fig. 4).

If the circulation is restored when the record has reached the base line (*d*, fig. 4), the functional activity reappears in stages resembling those seen at the beginning of stimulation,—there is a peak followed by a decline to a plateau. If during the period of increasing responsiveness, which follows exclusion of the blood supply, the circulation is restored, the response rapidly falls until it reaches the same level as before (*b*, fig. 4).

This increased ganglionic response during the first minutes of acute anemia at high-frequency stimulation has not been observed in every case. When it was missing (a decreased response being the only effect of the suppression of blood supply) the fact was noted that the animal had been given an unusually large dose of dial. Also this first phase was often missing at the end of a long experiment when the ganglion showed signs of fatigue. The phenomenon when present could be suppressed by giving an extra dose of dial. Thus, although not constantly observed, the initial increased response when acute anemia is established may reasonably be regarded as the normal reaction of the ganglion in good physiological conditions.

C. Discontinuous stimulation. The preganglionic fibers were stimulated

at tetanizing frequency for 5 sec. every minute or every half-minute. Thus time enough was given to allow the record almost to reach the base line during the interval between two stimulations.

During the first 3 or 5 minutes following suppression of the circulation the record no longer reached the base line, while the response to each stimulation showed a slight tendency to decrease (see fig. 5). Then the response decreased more markedly and the record gradually approached the base line between successive stimulations. The general pattern of this effect is not influenced by the frequency of stimulation and remains the same with frequencies lower or higher than 20 per sec.

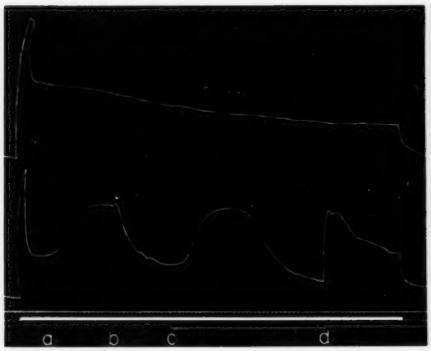


Fig. 4

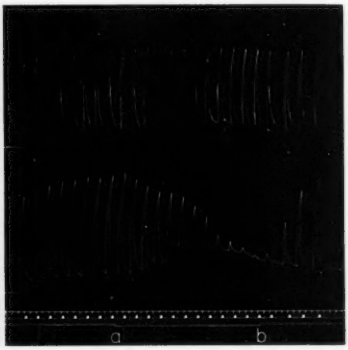


Fig. 5

Fig. 4. Increase of the response of the nictitating membrane to high frequency (40 per sec.) of stimulation of the preganglionic nerve when the blood supply to the ganglion is completely suppressed (between *a* and *b* and between *c* and *d*).

Fig. 5. Prolongation of the discharges from the superior cervical ganglion [in response to brief (10 sec.) tetanic stimuli (8 per sec.) applied every minute] when the blood supply was entirely suppressed between *a* and *b*.

Discussion. The changes observed in the contraction of the n.m. are clearly of ganglionic, not of peripheral origin. It should be remembered that ligation of one common carotid artery does not produce any noticeable change in the contraction of the n.m. This recognized fact was confirmed by stimulating the n.m. through its postganglionic nerve supply. If both the external and the internal carotids have been tied at the beginning of the experiment, clamping of the common carotid induces changes in the circulation of the ganglion only when other parts of the cephalic circulation are excluded. Furthermore, the circulatory alterations induced on the side under experiment have always been found to have no effect on the control side.

The period of increased ganglionic activity in the early stage of acute anemia, that was observed only during high frequencies of stimulation, was at first thought to be demonstrable exclusively in that condition. Reasons will be given for believing that it is a more general phenomenon. If the view is taken that transmission of nerve impulses through ganglionic synapses involves the liberation of a chemical mediator (Kibjakow, 1933), it is reasonable to inquire whether the phase of increased activity is caused by a rise in the concentration of the mediator, produced either by increased liberation or by slower destruction. Evidence on this point can be obtained by using pharmacological agents which modify the concentration of acetylcholine and by observing the consequent changes in the phenomenon. In present circumstances the possibilities of this procedure were restricted because the circulation was interrupted and the drugs, therefore, could not be brought to the ganglion sharply at a definite time; only relatively long-lasting pharmacological agents could be expected to have effects.

Prostigmin was utilized to protect acetylcholine from rapid destruction in cats previously atropinized (1 mgm. per kgm.). Different doses of prostigmin were used (from 1 to 3 mgm. intravenously). The common carotid was clamped after different intervals following the injection. No noticeable change in the effects of acute anemia was observed. In consideration of the relatively unfavorable conditions in which this test could be applied, it seems reasonable to conclude that the results do not support or exclude the possibility of an increased concentration of acetylcholine during the phase of increased ganglionic activity.

Another way of securing evidence was found in the experiments in which the ganglion was stimulated through preganglionic impulses aroused by a succession of short tetani at regular intervals. Time enough was given between two stimulations for destruction of any of the mediator that was liberated at the ganglion. In these circumstances a slowing of the destruction would not allow its entire disappearance before the next stimulation. This effect, even if slight, would be manifest by after-discharge from the ganglion between stimuli and, hence, by successive prolongations of the period of relaxation. If the s.c.g. is stimulated this way, suppression of the blood supply results first in a notable elevation of the base line above the initial level (see fig. 5). Slowing of destruction of the mediator, therefore, might be a possible explanation.

The long duration of the phenomenon (2 to 3 min.), however, does not accord well with what is known of the rapid destruction of acetylcholine. It seems more plausible that a more lasting adjuvant of excitation accumulates, e.g., K ions. Accumulation of K ions has already been suggested as the possible cause of post-tetanic increase of the ganglionic response to single shocks (Cannon and Rosenblueth, 1937). Bronk and

Larrabee (1937) observed that the post-tetanic increase of the electrical response is more marked in the non-circulated s.c.g. than in the normal. It would be worth while to examine more closely these results which might be found to be closely related.

A difference in the effects of acute anemia, according to the frequency of stimulation, is only apparent. When a succession of short tetani is applied to preganglionic fibers the reaction of the s.c.g. to acute anemia is the same at high and at low frequencies. In both cases accumulation of a stimulating substance seems likely during the period preceding that of decreased responsiveness.

During continuous stimulation maximal shocks were used. An increase in the response could be obtained only if some ganglion cells were idle as in the fatigued condition with stimulation at a high frequency. Such an increase would not occur when, at lower frequencies, all the fibers would be active and the n.m. maximally contracted. Thus may be explained the phase of increased ganglionic activity, observed in the records only when high frequency of stimulation was used. It was noted that the more marked the fall in the ganglionic response after the initial peak, the higher was its rise during the period of anemia. A diphasic reaction to acute anemia, therefore, seems to be general in the s.c.g.—a phase of increased activity preceding a phase of depression.

SUMMARY

The effects of acute anemia on the superior cervical ganglion were observed in cats under dial anesthesia during stimulation of the preganglionic fibers by maximal shocks, with the nictitating membrane (n.m.) used as an indicator.

In contrast to the cells of the central nervous system the sympathetic ganglion cells show a striking resistance to deprivation of blood. A very slight blood supply (e.g., that furnished by the minute artery running along the trunk of the vagus) is sufficient to keep the ganglion in normal activity. Complete suppression of the blood supply results gradually in a complete disappearance of activity of the ganglion; a partial reduction results in a decrease of activity (figs. 1 and 2).

After the start of total anemia, disappearance of activity takes from 10 to 15 minutes to become complete; it begins to reappear as soon as the circulation is restored, but the increase of functional response progresses more rapidly than the gradual decrease (fig. 1).

Complete deprivation of blood as long as 70 minutes may be followed by a large degree of functional recovery (fig. 3).

If the ganglion is being stimulated at a frequency higher than 20 per sec., suppression of the blood supply induces, during the first 2 or 3 minutes, an increased ganglionic response (see fig. 4).

If the ganglion is stimulated by a succession of short tetani, at a high or a low frequency, a slowing of the relaxation of the n.m. after each stimulus occurs during the first 2 or 3 minutes after the blood supply is excluded (see fig. 5).

The last two observations are discussed and it is considered likely that they result from accumulation of some stimulating substance, perhaps K ions, during the early period of anemia.

I wish to express my thanks to Dr. W. B. Cannon, who suggested and directed this work, and to Dr. A. Rosenblueth, for his valuable help.

REFERENCES

BRONK, D. W. AND M. G. LARRABEE. Proc. Am. Physiol. Soc., 1937.
BROWN, G. L. AND W. FELDBERG. J. Physiol. **88:** 265, 1936.
CANNON, W. B. AND I. R. BURKET. This Journal **32:** 347, 1913.
CANNON, W. B. AND A. ROSENBLUETH. Ibid. **119:** 221, 1937.
ECCLES, J. C. J. Physiol. **85:** 202, 1935.
FELDBERG, W. AND J. H. GADDUM. Ibid. **81:** 305, 1934.
FELDBERG, W. AND A. VARTIAINEN. Ibid. **83:** 103, 1934.
HERING, H. E. Pflüger's Arch. **99:** 253, 1903.
KIBJAKOW, A. W. Ibid. **232:** 432, 1933.
LANGENDORFF, O. Physiol. Centralbl. **5:** 327, 1891.
Pflüger's Arch. **56:** 522, 1894.
LANGLEY, J. N. J. Physiol. **15:** 176, 1894.
Ibid. **27:** 224, 1901.
ORÍAS, O. This Journal **102:** 87, 1932.
ROSENBLUETH, A. AND W. B. CANNON. Ibid. **119:** 221, 1937.
SCHRÖDER, R. Pflüger's Arch. **116:** 600, 1907.
TUCKETT, I. J. Physiol. **33:** 77, 1905.

ON THE EXISTENCE OF SECRETORY NERVES IN THE VAGI FOR AND THE REFLEX EXCITATION AND INHIBITION OF BILE SECRETION

CARLOS A. TANTURI[1] AND A. C. IVY

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago

Received for publication July 31, 1937

It is generally taught that secretory nerves for the formation of bile are not present in the vagi. This teaching is based principally on the observation of Heidenhain who found that stimulation of the vagi does not augment bile formation. Arthaud and Butte (2) reported that electrical excitation of the peripheral end of the vagus in the neck inhibited, and of the intact nerve or of its central end stimulated the formation of bile in the rabbit and dog. Eiger (3) stimulated the vagus above the diaphragm in the dog and observed an increase in the formation of bile. He stimulated the nerve for several minutes, and then after a period of "rest" stimulated again, and so on. This work has been either overlooked or considered not to be convincing (4). Yet, Yanagawa (5) has reported a few experiments in which pilocarpine intravenously (3 mgm.) in the dog augmented bile flow 100 per cent or more, and atropine diminished bile flow. The inhibitory effect of atropine on bile flow was also reported by Prevost and Binet (6). These observations may not have been considered to be convincing because in no instance was the gastro-intestinal tract removed to eliminate changes occurring there which might influence the formation of bile.

It occurred to us that the method used by Pavlov (7, 8) to demonstrate "secretory nerves" in the vagi for the pancreas might be employed to ascertain if the vagi influenced bile formation. This method consists in stimulating the peripheral end of the vagus after it has been sectioned four or five days and when the cardio-inhibitory nerves have degenerated.

METHODS. Either the right or left vagus nerve was aseptically sectioned in the neck under ether anesthesia in dogs (vago-sympathetic trunk), cats and monkeys (Rhesus). Several days later, the time varying in the different animals, the animal was anesthetized with pentobarbital (Nembutal), the cystic duct was tied, the common duct was cannulated, and the carotid artery prepared for a blood pressure record. The stimu-

[1] Becado, Comision Nacional de Cultura, Buenos Aires.

lating current was obtained from a Harvard inductorium receiving current from two ordinary dry cells.

RESULTS. *Stimulation of the undegenerated peripheral end of the right vagus above the diaphragm.* We first performed experiments on five dogs in which the undegenerated right vagus above the diaphram was sectioned and its peripheral end placed in shielded electrodes for stimulation. After obtaining a suitable control over a period of 30 minutes or more, the nerve was stimulated for five minutes; after a period of 10 minutes the vagus was again stimulated. In these experiments the nerve was first stimulated twice with the secondary coil set at 6.5 cm. This setting was then increased or decreased to ascertain if a stronger or weaker current might be effective. Current interrupted at various frequencies was also used.

No effect was observed in one dog; a moderate (25 50 per cent) decrease in bile formation was observed in three. In one animal a definite increase in flow resulted.

Stimulation of the degenerated nerve in the neck. In all the dogs used in this experiment, the vagus was permitted to degenerate for five days, since this time was found in a study on the pancreas (8) to be close to the optimal period for the dog. The right vagus was sectioned in ten and the left in two dogs.

In all animals the first current used was obtained with the inductorium set at 6.5 cm. It was applied for five minutes. Generally this strength of current was applied a second time after the bile flow returned to the control level. Then, various strengths of current were used, some continuous and some interrupted, half second on and half second off. The current delivered with the secondary coil at 6.5 cm. and applied continuously gave the best results. When the stimulus was applied the blood pressure generally rose slightly (fig. 1). In two of twelve animals the application of the first stimulus caused a slight fall (20 mm. Hg), followed by a slight rise in blood pressure. As will be noted in table 1, the current was applied repeatedly in several instances. In such instances the current was applied for five minutes and then repeated at from seven to eleven minute intervals three or four times during a one-hour period.

The essential results are recorded in table 1. It is to be noted that nine of ten dogs showed an increase in bile formation on stimulation of the degenerated right vagus nerve. The type of response is shown for two of the dogs in figure 1. Referring to the figure, it will be noticed that bile flow was decreased for the major portion of the period of stimulation. This decrease is associated with the period of slight elevation in blood pressure. Occasionally as the blood pressure started to rise a drop of bile appeared to be squeezed out. No decrease in bile flow during the period of stimulation was observed in only two of twelve dogs.

To avoid the possibility that the increase in bile formation might be due to a discharge of acid gastric contents into the duodenum, the pylorus was obstructed in four of the ten experiments in which the right vagus was stimulated. The secretory effect was still obtained.

To avoid the possibility that the secretory effect might be secondary to some change in the gastro-intestinal tract, the small intestine, duodenum, pancreas, and spleen were removed. The stomach was not re-

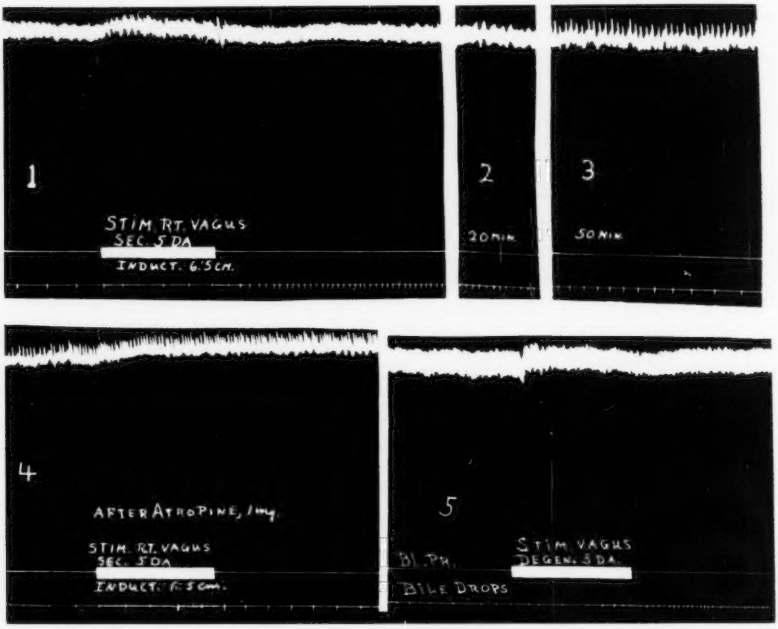


Fig. 1. Tracings 1, 2, 3 and 4 show the effect of stimulation of the peripheral end of the right vagus nerve five days after sectioning on bile secretion. In tracing 4 no augmentation of bile formation occurred after atropine. Tracing 5 shows the immediate response of a second dog to stimulation of the degenerating vagus. Note that only minor changes in blood pressure occurred.

moved, because we did not desire to disturb the vagal nerve supply of the liver; instead we tied the vessels supplying the stomach. Two experiments of this nature were performed; on stimulating the vagus, an increase in bile formation occurred (table 1). Further, in two dogs the vagus branched along the lesser curvature and distal to the point at which the hepatic branches of the vagus nerve trunk were stimulated. Although the pyloric antrum contracted, the formation of bile was not augmented.

TABLE 1

Showing the results of stimulation of the peripheral end of the vagus nerve sectioned five days previously in the dog and monkey

DOG NUMBER	STIMULATION	RIGHT VAGUS SECTIONED		ACTUAL INCREASE† FOLLOWING STIMULATION	REMARKS
		Rate of Control flow*	Maximum rate of flow after stimulation		
		cc. per hour	*cc. per hour*	*cc.*	
1	1	2.7	13.2	3.3	Single 5 minute stimulation. Induct 6.5 cm.
	2	2.5	6.5	1.0	Single 5 minute stimulation. Induct 6.5 cm.
2	2	5.0	16.1	2.2	Single 5 minute stimulation. Induct 6.5 cm.
3	1	6.0	8.4	0.5	
4	Repeated	2.4	14.0	5.4	Three 5 minute stimulations 7-11 minutes apart
5	1	2.4	5.4	0.5	
	Repeated	2.4	7.8	3.5	Three 5 minute stimulations in 40 minutes
6	Repeated	4.0	9.0	3.0	Three 5 minute stimulations in 40 minutes
	Single	4.6	8.4	1.2	
	Single	4.6	7.8	1.2	
7	1	3.6	7.1	1.0	Before evisceration
	2	2.0	4.0	0.5	After evisceration
8		3.4	Obtained only inhibition with various stimulations		
9	1	5.2	10.9	2.4	Before evisceration
	2	2.4	4.8	1.6	After evisceration
10	1	7.8	10.2	0.4	Right stellectomy
	2	7.8	10.2	0.4	
		Left vagus sectioned			
11	1	3.6	6.0	0.5	
	Repeated	3.6	6.6	1.7	Three 5 minute stimulations in 40 minutes
12	1	3.6	5.4	0.3	
	2	3.0	5.8	1.2	
		Monkey (Rhesus)			
M.1	Repeated	1.8	2.7	0.5	Body weight 4 pounds
M.2	Repeated	2.7	4.0	0.9	Body weight 6.5 pounds

* Calculated on the basis of the maximum 5 minute period.

† Actual volume increase that resulted from stimulation. For example, dog 1 secreted 2.7 cc. in 1 hour before stimulation; after stimulation 6.0 cc. were collected in 1 hour, giving an increase of 3.3 cc.

To avoid entirely the slight rise in blood pressure that occurred on stimulating the nerve (vago-sympathetic trunk), the right stellate ganglion was removed in one animal at the time the vago-sympathetic trunk was sectioned. This was also done to avoid the possibility that stimulation of the vago-sympathetic trunk might increase bile formation reflexly via the sympathetic nerves. Only one such experiment was performed. Although the secretory response was not marked, secretion was definitely increased (table 1). (Experiments will be described later which annul the sympathetics as an excitatory secretory pathway.)

In two animals the left vagus was sectioned and stimulated five days later. A secretory response was obtained (table 1).

Results in chronic fistula animals. In two animals with a recently prepared chronic biliary fistula, the right vagus nerve was sectioned. Five days later the peripheral end of the sectioned nerve, which had been placed just beneath the skin, was exposed without anesthesia and stimulated. The animal was not distressed, as has been previously reported by Pavlov. The bile output was measured by connecting the drain tube to a 1 cc. pipette graduated to 0.01 cc.; readings were made at five minute intervals. The results of the first stimulation on the first dog were as follows: Control 5 minute periods yielded: 0.25, 0.35, 0.35, 0.35, 0.35 cc. Nerve stimulated for 5 minutes; flow during, 0.30 cc. Flow after stimulation: 0.45, 0.40, 0.60, 0.80, 0.50, 0.3, 0.20 cc. The control 25 minute period yielded 1.65 cc., and a similar period after stimulation 2.75 cc., an increase of 66 per cent. A second experiment yielded: Control 5 minute periods: 0.09, 0.06, 0.09, 0.04, 0.09 cc. Five minute period of stimulation: 0.11 cc. Following: 0.16, 0.16, 0.16, 0.18, 0.14, 0.14, 0.14, 0.17, 0.17, 0.15, 0.13, 0.13, 0.12, 0.09, 0.10, 0.07, 0.07, 0.09, 0.09, 0.06. The control 25 minute period yielded 0.37 cc., and the following 25 minute period 0.80 cc., an increase of 116 per cent. It is rather obvious that the increase was not due to a "squeezing out" effect; but, to verify an actual increase in volume output, the bilirubin content of the bile before and after stimulation was estimated. The bilirubin content before was 507 mgm. per 100 cc., and after 255 mgm., a dilution of 98 per cent. The next day bile was collected for one hour and then 5 mgm. of pilocarpine hydrochloride were injected subcutaneously. The bile collected during the control hour amounted to 2.6 cc., during the hour after injection 2.6 cc., the second hour 1.4 cc., and the third, 2.9 cc. This was not considered a decisive result. A second experiment with pilocarpine was performed, the results of which were no more decisive. Similar experiments were performed on a second dog. The control before stimulation of the nerve yielded: 0.09, 0.12, 0.14, 0.15, 0.16 cc.; during stimulation, 0.15 cc.; after stimulation, 0.20, 0.20, 0.14 cc. The nerve was then stimulated again for 5 minutes; during stimulation 0.11 cc.; after stimulation, 0.18, 0.17, 0.18, 0.19, 0.24,

0.25, 0.19, 0.24, 0.25, 0.19, 0.12, 0.10, 0.07 cc. A third stimulation of the nerve again increased the output, but not above a rate of 0.18 cc. per five minute period. The 25 minute control period yielded 0.66 cc. of bile; the maximum post-stimulation period, 1.17 cc., a 77 per cent increase. A fourth stimulation also resulted in an increase. The results obtained from the subcutaneous injection of pilocarpine in this animal were also indecisive, which is attributed to the nauseating effects of the drug. Vomiting is always associated with an inhibition of bile formation.

Monkeys. The right vagus nerve was sectioned in the neck in two small monkeys. Five days later the nerve was stimulated and the rate of secretion was increased in both (table 1). The cardio-inhibitory fibers had degenerated and a cardio-accelerator effect was not observed. It is possible that a greater secretory effect may have been obtained if less time had been permitted for degeneration. Inhibition of secretion was not observed during the period of stimulation in either monkey. Experiments on monkeys were not extended because the animals were not available.

Cats. In three cats the right vagus was sectioned in the neck. Five days later the nerve was stimulated. In two a slight but insignificant increase in the rate of secretion occurred after repeated stimulation. In the other no change occurred. The cats responded normally to bile salt. Inhibition of secretion resulted in only one of the three cats. Thinking that possibly five days of degeneration was too long, the degenerating right vagus nerve of two cats was stimulated three days after section. Stimulation of bile formation was not obtained; inhibition was obtained in both.

Effect of atropine on the secretory response. It was found that atropine sulphate given intravenously definitely reduces the secretory response to stimulation of the degenerating nerve. One or 2 mgm. was adequate to abolish the response; however, in dog 2, 4 mgm. did not entirely abolish the response. The drug when used alone caused a decrease of the spontaneous secretion, but it could not be distinguished clearly from the natural decline in the formation of bile that occurs during such an experiment.

The effect of other drugs. The effect of eserine salicylate on the secretory response to nerve stimulation was studied in three dogs. One milligram of the drug given intravenously had no effect on the spontaneous secretion of bile or on the response to stimulation of the nerve. Two milligrams decreased the formation of bile, but did not significantly alter the secretory response to stimulation of the nerve.

Mecholyl (acetyl-β-methylcholine chloride) given subcutaneously in doses of 0.02 mgm. to 0.1 mgm. per kilo body weight every ten minutes for one hour had no effect on the spontaneous formation of bile. One-tenth milligram given intravenously slightly inhibited the formation of

bile. Acetylcholine was used in only two animals; only inhibition was observed. We consider these experiments insufficient to determine the effects of various doses of these drugs. The results are mentioned only because of their possible interest.

TABLE 2

Pilocarpine subcutaneously in anesthetized animals

DOG NUMBER	CONTROL RATE OF FLOW	AFTER PILOCARPINE		DURATION OF EFFECT
		Maximum rate	Excess secretion over a 1-hour period	
	cc. per hour	*cc. per hour*		*minutes*
1*	1.5	4.2	2.5	120+
2*	4.0	6.0	1.5	60+
3*	1.4	3.6	2.0	120+
4*	1.7	7.5	2.0	120+
5	2.7	5.7	2.5	120+
6	2.4	4.2	1.5	60+
7	2.4	6.0	2.45	120+
8	2.1	3.6	1.3	60+
Removal of the G-I tract, spleen and pancreas				
9	2.25	3.5	0.5	45
10†	1.0	1.2	0	
11	0.5	3.4	1.75	60+
Cat 1	1.2	2.5	0.4	45
Prostigmine intravenously, 0.05 mgm. per kilo intravenously				
1	4.8	5.6	0.5	47
2	3.0	13.2	5.5	35
3	2.8	4.8	1.1	25
4‡	2.2	0	0	
5‡	3.2	0	0	
6‡	5.4	7.2	0.3	45
7‡	3.6	4.8	1.0	60+
8	4.8	8.4	2.0	60+

* Atropine sulphate, 1 mgm. intravenously promptly returned the flow to the control level.

† Although this dog was not stimulated, the rate of bile formation did not decline and was at the "pre-evisceration" control level 3 hours later.

‡ Both vagi sectioned.

Pilocarpine, when given in a dose of 1 mgm. intravenously to three dogs, weighing 10 to 15 kilos, first caused an inhibition of the spontaneous secretion which lasted for from 5 to 20 minutes, and then definite stimulation occurred. To avoid the vascular effect of the intravenous injection,

5 mgm. of pilocarpine were injected subcutaneously. This experiment was performed in eight dogs. The results are recorded in table 2. A primary inhibition of bile formation was observed in only one of the dogs. The stimulating effect in these experiments continued for from 45 minutes to more than 2 hours. To avoid the possibility that the increase in flow of bile was secondary to changes in the stomach and intestine, we removed these organs along with the spleen and pancreas. This was done in three dogs (table 2); the bile flow of two was increased; in the other (dog 9), an increase did not occur, but the usual decline in formation of bile, as such experiments progress, was not observed during a period of two hours after the injection.

Pilocarpine, 4 mgm., was injected subcutaneously into four cats. The

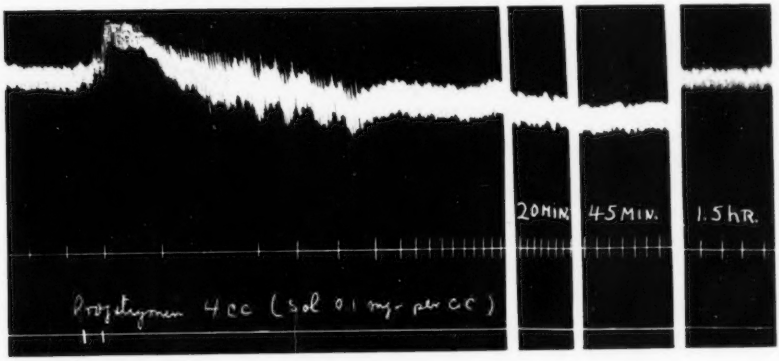


Fig. 2. This tracing shows the primary inhibition and secondary stimulation of bile formation after the intravenous injection of 0.05 mgm. prostigmine per kilo body weight.

secretion of bile was increased in only one; it was increased by one hundred per cent and the effect continued for more than one hour.

Prostigmine was injected intravenously in a dose of 0.05 mgm. per kilo, a dose which is known (10) to stimulate pancreatic secretion to a very definite but variable extent. Following the injection, a primary inhibition of bile secretion lasting from five to ten minutes occurred; this was then followed by stimulation in six of eight dogs. In the dogs which were not stimulated (dogs 4, 5), a marked and prolonged cardio-inhibition resulted; but in these dogs smaller or larger doses repeated at intervals of one hour did not stimulate (fig. 2).

Stimulation of the intact vagus in the neck. In the experiments on stimulating the right vagus above the diaphragm, the duration of the stimulus was five minutes. It occurred to us that we might obtain different results (less operative procedure) by stimulating the vagus in the neck for a longer

period than five minutes, so that the stimulus might be applied after escapement of the heart from cardio-inhibition.

This was done in eight dogs. During the period of marked alterations in blood pressure resulting from the stimulation of the intact vagus, the flow of bile was usually irregular and was decreased. After the heart escaped, an increase in the flow of bile occurred in every animal. The degree of increase and its duration is shown in table 3 (fig. 3).

The fact that a secretory effect could be demonstrated by stimulating the vagus in the neck rendered it simple to determine whether the formation of bile was influenced reflexly via the vagus. It was first shown that bile formation could be increased by stimulating the right or left intact vagus. It was then found that the stimulation of the central end of one vagus with the other intact would increase bile formation. But, after section of both vagi, the stimulation of the central end of either vagus

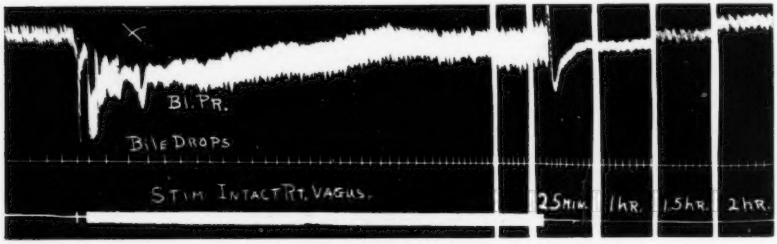


Fig. 3. This tracing shows the augmentation of bile formation that occurred after stimulation of the intact right vagus nerve.

failed to increase bile formation; in fact bile formation was inhibited (via sympathetics in the spinal cord) in all the animals but one (dog 4) in which no change occurred.

The intact right and left vagus were stimulated in six cats. Various strengths of current were used. (The placement of secondary coil varied from 10 cm. to 5 cm.) Stimulation of the central and peripheral ends of the sectioned right or left vagus was also performed. In no instance was a significant increase in bile formation observed. Inhibition of secretion was observed, but this may have been due to vascular changes, although the inhibitory effect outlasted the vascular changes.

In view of the fact that the functional sympathetic innervation of the cat's submaxillary gland differs from that of the dog, we stimulated the peripheral end of the right splanchnic nerve in the cat with various strengths of current and for periods of various lengths. As in the dog, only inhibition of secretion was obtained.

TABLE 3

Stimulation of the non-degenerated vagus in the neck

DOG NUMBER	PROCEDURE	RATE OF CONTROL FLOW	MAXIMUM RATE OF INCREASE AFTER STIMULATION	ACTUAL INCREASE DUE TO STIMULATION	DURATION OF INCREASE IN FLOW	REMARKS
		cc. per hour	*cc. per hour*	*cc.*	*minutes*	
1	Intact right vagus	1 8	4 2	2 2	60+	Stimulation 17 minutes; inhibition during
	Intact right vagus	2 0	3 8	1 7	40	Stimulation 17 minutes; inhibition during
	Central right vagus	2 0	4 2	1 0	20	Stimulation 15 minutes; inhibition during
	Left vagus cut	2 0	0	0	0	
	Central left vagus	2 0	Inhibition only			Stimulation 15 minutes
2	Intact right vagus	4 8	−2 4			Stimulation 10 minutes; inhibition only
	Intact right vagus	2 4	4 8			Flow returned only to control rate
	Intact left vagus	4 6				Animal succumbed
3	Intact right vagus	4 7	6 6	1 5	50+	Stimulation 25 minutes; inhibition during
	Central right vagus	4 8	6 0	0 5	15	Stimulation 20 minutes; inhibition during
	Peripheral right vagus	4 8	6 0	0 6	15	Stimulation 10 minutes; inhibition during
4	Intact right vagus	3 0	6 0	2 0	45+	Slight inhibition during stimulation 22 minutes
	Cut left vagus	4 4				No effect
	Intact right vagus	4 4	7 2	1 4	55	Stimulation 12 minutes
	Right vagus cut	3 6	−0 4	−0 4		
	Central right vagus	3 2	0	0		Inhibition during stimulation 10 minutes
	Peripheral ends, both	3 2	4 8	0 6		Stimulation 18 minutes
5	Intact right vagus	7 0	12 0	3 0	40	Inhibition during stimulation 20 minutes
	Cut right vagus					Inhibition
	Intact left vagus	6 0	10 2	2 0	45	Stimulation 20 minutes
	Cut left vagus	6 2	0	0	0	
	Central right vagus	6 2	0	0	0	Inhibition during
6	Intact right vagus	1 1	4 0	1 4	60+	Inhibition during stimulation 20 minutes
	Central right vagus	2 8	4 0	0 5	20	Stimulation 15 minutes; slight inhibition during
7	Intact right vagus	4 8	12 6	5 0	120+	Stimulation 25 minutes; slight inhibition
	Cut right vagus	6 6	0	0	0	Cut 2.5 hours later during
	Central right vagus	6 6	7 7	0 4	15	Stimulation 15 minutes; inhibition during
	Intact left vagus	6 6	10 1	1 7	35	Stimulation 20 minutes; inhibition during
	Cut left vagus	6 6	0	0	0	
	Central left vagus	6 6	−2 2	−2 2	60	Returned to control level inhibition only
8	Central left vagus	1 8	3 6	0 8	45	Stimulation 20 minutes; inhibition during
	Intact right vagus	2 2	4 8	1 2	60+	Inhibition during, only
	Cut right vagus	2 2	0	0	0	
	Central left vagus	2 2	0	0	0	Inhibition during, only

Similar experiments were performed on five rabbits. Only in two (2 rabbits) instances (secondary coil 9 cm.) was bile flow increased; in one, 33 per cent increase followed by a return to the control rate, in the

other a 25 per cent increase followed by a compensatory decrease after the stimulation. These increases appeared to be due more to a motor effect on the ducts rather than to a true increase. A decrease of secretion was observed in the other tests on the two rabbits just referred to and in all tests on the remaining three.

Incidental observations on acholia. During the course of this and other work on bile secretion, seventy-two dogs have been used. Of these four developed acholia during the progress of the experiment and failed to secrete bile after the intravenous injection of 2, 5 and then 10 cc. of a 20 per cent solution of the sodium salt of dehydrocholic acid. One of the animals had an acute cholecystitis, but bile, normal in color, flowed after occluding the cystic duct and cannulating the common duct. After stimulating the vagus nerve in the neck, which had been sectioned five days previously, the bile flow completely ceased and could not be started again with bile salts. A second animal also had an acute cholecystitis. Bile, normal in color, flowed from the cystic duct until a cannula was placed in the colon when the flow ceased and could not be started with bile salt. In the third and fourth animals cholecystitis was not present. Bile flow ceased in one after the dissection of the hepatic plexus and in the other after the cannulation of the colon; in neither could the flow of bile be started with bile salt. Cinchophen which, by weight, is a somewhat more potent cholagogue in the dog than dehydrocholic acid (11), also failed to cause secretion of bile in these four animals after bile salt had been tried. (Cinchophen does not stimulate bile formation in the rabbit. In the cat it stimulates slightly.) The cannula in the common duct was not occluded; if such had been true, the bile ducts proximal to the cannula would have been distended with bile. In all of the animals the blood pressure was above 120 mm. Hg. The mechanism responsible for the production of the acholia is problematical, but we suspect that the liver of these animals was profoundly sensitive to inhibitory nervous influences.

Discussion. On the basis of the evidence obtained on the dog, there is no doubt concerning the existence of fibers in the vagi which exert an inhibitory and excitatory *secretory effect* (bile volume output) on the liver. The right vagus appears to be more effective in this regard than the left. The existence of a specific, excitatory-secretory effect is deducible even when the intact vagus in the neck is stimulated. Yet, because of the marked circulatory effect, which precedes the augmentation of bile flow, and the presence of the gastro-intestinal tract and other viscera, which when activated by the vagus may secondarily augment bile formation, such a deduction might be questioned. Both of the objections, however, are avoided by stimulating the five-day-degenerated vagus peripherally in the "eviscerated" dog.

The inhibition of bile formation, which usually occurs early during the stimulation of the five-day-degenerated vagus peripherally, must be due to a specific inhibitory effect, because the vascular change is so slight as to almost annul the idea that the inhibition is due to an increase in intrahepatic vascular pressure. Yet, if marked vascular changes do occur, as in the experiments on stimulating the non-degenerated vagus, the inhibition of secretion is much more marked.

Our failure to observe augmentation of bile output in four of five dogs on stimulating the non-degenerated vagus above the diaphragm may have been due to several factors. First, the period of stimulation may not have been of sufficient length, this we think to be very likely. Second, as is sometimes true of the stomach and pancreas, the inhibitory effect of the undegenerated nerve may have predominated (8). Augmentation of gastric and pancreatic secretion is obtained more uniformly on stimulating the degenerating nerve than the intact nerve, because it is believed that the inhibitory fibers degenerate faster than the excitatory-secretory fibers. Third, early degeneration may result in the sensitization of the excitatory-secretory nerve endings in the liver. It is to be noted (table 1) that every dog with a degenerating vagus had a rather copious spontaneous secretion.

The evidence that the vagus of the dog contains sensory nerves which reflexly augment the formation of bile, as long as the other vagus is intact, is clear. Whether this reflex vagal augmentation of bile secretion is due directly to the excitation in the medulla of the cells of the excitatory secretory nerves or indirectly to changes in the stomach and intestine is uncertain; this is because we have performed no experiments on "eviscerated" animals in regard to this point. Yet, the facts that the vagus contains fibers which have a specific secretory effect on the liver (results of experiments on the degenerating nerve), even in the absence of the gastro-intestinal tract, spleen and pancreas, and that the stimulation of the central end of one vagus with the other intact increases bile secretion, constitute very strong presumptive evidence for the direct effect of the sensory impulses on a secretory center for bile formation in the medulla.

It has been previously (9) shown that stimulation of the splanchnic nerves markedly decreases the formation of bile. This is mentioned because inhibition of bile formation was observed when the central end of one vagus was stimulated with the other sectioned. The vascular change during the stimulation was quite marked; the blood pressure increased from 30 to 50 mm. Hg and then gradually returned toward the control level as the stimulation progressed. Such a vascular change (9) might account for the inhibition; but it could not reasonably account for

the inhibition which continued to be manifest for one-half hour after cessation of the stimulation of the nerve in three (dogs 1, 4, 7; table 3) of the five dogs. Thus, it is believed that the inhibition observed on stimulation of the central end of one vagus with the other sectioned was due to a reflex inhibitory effect (vaso-constriction) on the liver via the splanchnic nerves. We did not section the splanchnic nerves to prove this contention, because section of the splanchnic nerves *per se* increases bile formation (9). Vasodilator fibers for the liver are not present in the vagi according to Griffith and Emery (12) and the more recent work of McMichael (13) who reviews that literature on the subject.

The situation in regard to the secretory innervation of the liver in the dog is quite analogous to that of the pancreas. From the experience of one of the authors (A. C. I.), gained by a study of the secretory nerves of the pancreas (8), it is believed that the secretory effect exerted by the vagi on the liver is of longer duration as a rule. It, therefore, follows that a small "psychic" secretion of bile should be obtained when the adequate experiment is performed, as is true for the pancreas.

The parasympathomimetic drug used which most uniformly stimulated bile formation in the dog was pilocarpine. Our observations on mecholyl, acetyl choline, and eserine were inadequate. The primary inhibitory and secondary augmentative effect of pilocarpine and prostigmine given intravenously is analogous to the effects of stimulation of the vagus and constitutes pharmacologic evidence for the existence of two types of parasympathetic fibers which influence bile formation.

Our results indicate that excitatory-secretory fibers in the vagus for bile formation are not present in the cat and rabbit. The fact that one cat responded to pilocarpine and an apparent increase occurred on the stimulation of the intact vagus in two tests in the rabbit, suggests that they may exist, and might be demonstrated by some method of approach not tried by us. An inhibitory secretory effect in the cat and rabbit is obtained by stimulation of the vagus or splanchnic nerves. The failure to obtain a secretory effect in the cat and rabbit immediately brings to mind the difference that exists between the cat and dog in regard to the muscle fibers which are present in the hepatic veins of the dog and not in the cat. This fact suggests that the inhibitory and secretory effects observed, even in the experiments with the degenerated nerve, may be due to a vascular change in the liver. If so, the vascular change responsible for the augmentation of secretion must have been of prolonged duration and of such a nature as not to affect systemic blood pressure.

In connection with our experimental results, those of Snyder (14) are of interest. He found that a "vagal-like substance" appears in the hepatic outflow after electrical stimulation of the vagus in the turtle.

CONCLUSIONS

1. A very definite excitatory-secretory *effect* on bile formation may be obtained in the dog and monkey, but not the cat and rabbit by stimulating the peripheral end of the vagus nerve in the neck five days after it has been sectioned. The secretory effect is not accompanied by a change in systemic blood pressure. During the period of stimulation an inhibitory secretory effect may be observed in the dog, monkey and cat.

2. Prolonged stimulation of the intact vagus nerve in the neck in the dog, but not the cat and rabbit, results in prolonged augmentation of bile formation after the heart has escaped from vagus inhibition. Augmentation of bile secretion may be obtained in the dog when the central end of one vagus is adequately stimulated when the other vagus is intact. Section of the second vagus abolishes the augmentative effect of stimulation of the central end of the other vagus. This demonstrates the existence of a reflex vagal augmentation of bile formation in the dog. With the vagi sectioned, the stimulation of the central end of one reflexly diminishes bile formation through paths in the spinal cord.

3. Pilocarpine and prostigmine in the dog first inhibit and then definitely augment the formation of bile.

4. The excitatory-secretory effects are obtained in dogs devoid of the gastro-intestinal tract, spleen and pancreas.

5. Motor nerve fibers exist in the vagi of the dog which exert an inhibitory and an excitatory effect on bile formation by the liver. The liver is quite analogous to the pancreas in regard to secretory innervation. Sensory fibers which reflexly excite bile formation by way of the vagus nerves and which reflexly depress bile formation (hepatic vasoconstriction) by way of the spinal cord, are also present in the vagi.

REFERENCES

(1) Heidenhain. Handb. d. Physiol. **5:** 270, 1883.
(2) Arthaud and Butte. Compt. rend. d. l. Soc. Biol. **2:** 44, 1890.
(3) Eiger. Ztschr. f. Biol. **66:** 229, 1915–16.
(4) Babkin. Die Aussere Sekretion der Verdauungsdrusen. 1928.
(5) Yanagawa. J. Pharmacol. Exper. Therap. **9:** 75, 1917.
(6) Prevost and Binet. Compt. rend. d. Acad. Sci. **106:** 1888.
(7) Pavlov. The work of the digestive glands. London, 1910.
(8) Crittenden and Ivy. This Journal **119:** 724, 1937.
(9) Tanturi and Ivy. This Journal **121:** 61, 1937.
(10) Crittenden. Unpublished work.
(11) Tanturi and Bradley. Unpublished work.
(12) Griffith and Emery. This Journal **95:** 20, 1930.
(13) McMichael. J. Physiol. **77:** 399, 1932.
(14) Snyder. This Journal **118:** 345, 1937.

THE BEHAVIOR OF THE DYE T-1824 WITH RESPECT TO ITS ABSORPTION BY RED BLOOD CELLS AND ITS FATE IN BLOOD UNDERGOING COAGULATION

MAGNUS I. GREGERSEN AND H. SCHIRO[1]

From the Department of Physiology, Harvard Medical School

Received for publication August 2, 1937

Exact information concerning whether or not a dye is taken up by the red blood cells is of considerable importance if the dye is to be used for determinations of plasma volume. From the results of various attempts to study the reaction between blood cells and such dyes as vital red (Keith, Rowntree and Geraghty, 1915; Harris, 1920; Lindhard, 1926), brilliant vital red (Hooper, Smith, Belt and Whipple, 1920) and trypan blue (Seyderhelm and Lampe, 1922) it is generally concluded that no absorption takes place, but even if due allowance is made for the limits of accuracy of the colorimetric methods employed in these tests, the evidence as a whole indicates that a small fraction (1 to 5 per cent[2]) of the dye added to whole blood in vitro fails to appear in the plasma separated by centrifugation (Erlanger, 1921). The purpose of the following experiments was to determine more exactly the extent of this loss and to find out whether it could be attributed to a combination of dye with the erythrocytes. Except 5 tests with brilliant vital red, the experiments were done with a pure preparation of the blue dye T-1824 (Hartwell and Fieser, 1936), which for reasons set forth elsewhere (Gregersen, 1937), can be determined spectrophotometrically with less chance of error than is true for any of the red dyes. Since T-1824 is an acid dye and therefore carries a charge of the same sign as that of the erythrocytes (Harris, 1920), and furthermore since it is slowly diffusible, as indicated by its inability to pass through collodion membranes as well as by its slow disappearance from the blood stream (Gregersen, Gibson and Stead, 1935), it seems unlikely that this dye either stains or penetrates the red blood cells.

[1] Research Fellow in Medicine, Department of Medicine, Harvard Medical School.

[2] One exception to this is the report by Lindhard (1926) that the red blood cells of the ox and dog take up vital red in large amounts (up to 48 per cent). His results on human blood, however, are in agreement with the general statement made above. Since there is some indication in his paper that he did not use the same dyestuff throughout, it is possible that the differences may be explained by the use of different dyes. It should also be pointed out that Lindhard's results are questionable since he apparently used water standards in his colorimetric determinations (Gregersen, 1937; Gregersen and Gibson, 1937).

Recovery of dye from whole blood. The tests were carried out by adding a known quantity of dye to a measured amount of blood (oxalated or defibrinated) and subsequently determining the amount present in the plasma (or serum) separated by thorough centrifugation. The procedure was simplified as much as possible to avoid errors and was briefly as follows: one-tenth cubic centimeter of an aqueous dye solution (usually 1 per cent) was transferred with the same Folin pipette to each of two volumetric flasks, *a*, containing 50 cc. of whole blood, and *b*, containing 25 cc. of plasma obtained by centrifuging another portion of the same blood. The simultaneous preparation of the standard solution *b* was deemed essential in order to rule out any possibilities of error arising from the use of plasma from another source. It was found that these standards could be prepared with an error of less than 0.5 per cent. After the contents of the two flasks had been mixed gently for at least 5 minutes, six or eight samples of the dye-containing blood were centrifuged for 30 minutes at 3000 r.p.m. in calibrated hematocrit tubes 10 cm. long and of 4 cc. capacity. The radius of the centrifuge head was 15 cm. The volume of plasma in *a* was calculated from the average of these hematocrit determinations. The plasma from each sample was then read in the spectrophotometer[3] against an identical dye-free plasma and the average value was used in calculating the amount of dye present in the plasma of flask *a* as compared with the amount present in the standard solution *b*. Experiments in which there was any suggestion of hemolysis (detected spectroscopically) were at once discarded since hemolysis of the unknown tends to mask the effect of loss of dye from the plasma.

The results of 5 experiments with brilliant vital red and of 8 experiments with T-1824 are summarized in table 1, from which it may be seen that the average amount of dye remaining in the cell residue (4.2 per cent) is the same for both dyes. The chances are that this would still be true for a larger group of experiments since in both cases the results fall within the same narrow range. Indeed, two-thirds of them are within about 1 per cent of the general average. Also it should be noted that the results in two experiments on human blood (12 and 13) are essentially the same as those obtained with dog blood. The question is: Do these observations necessarily mean that dye is taken up by the cells? Obviously this point cannot be decided without first ascertaining how completely the plasma and cells are separated by centrifugation.

Numerous investigations of the hematocrit method have shown that it is quite inadequate for accurate determinations of relative cell and plasma volume. For example, in two experiments with the nitrogen

[3] The optical density of brilliant vital red was determined at 500 mμ. T-1824 at 620 mμ.

method of Bleibtreu and Bleibtreu (1892), Keith, Rowntree and Geraghty (1915) found the relative plasma volumes to be 4 and 9 per cent higher than those obtained by centrifugation at 3000 r.p.m. for 20 minutes. The colorimetric hemoglobin method of Stewart (1899) also gives higher values than those obtained from the hematocrit determinations at similar speeds (Ponder and Saslow, 1930; Jenkins, 1932). More recently Oberst (1935) has demonstrated that the sodium content of red blood cells (man) separated by centrifugation is considerably reduced by washing the cell residue with prepared sodium-free serum or isotonic potassium chloride. His experiments indicate that approximately 3 per cent of the total plasma

TABLE 1

Recovery of dye from whole blood in vitro

Showing the difference (per cent) between the amount of dye added to whole blood in vitro and the amount recovered in the total quantity of plasma obtained by centrifugation (30 minutes at 3000 r.p.m., radius 15 cm.).

EXPERIMENT NUMBER	DYE	BLOOD	PER CENT OF DYE LOST	
1	Brilliant vital red*	Oxalated (dog)	4.0	
2	Brilliant vital red	Oxalated (dog)	6.1	
3	Brilliant vital red	Oxalated (dog)	3.9	4.2 Average
4	Brilliant vital red	Oxalated (dog)	2.7	
5	Brilliant vital red	Oxalated (dog)	4.3	
6	T-1824†	Oxalated (dog)	2.3	
7	T-1824	Defibrinated (dog)	3.5	
8	T-1824	Defibrinated (dog)	5.6	
9	T-1824	Defibrinated (dog)	7.0	
10	T-1824	Defibrinated (dog)	3.5	4.2 Average
11	T-1824	Defibrinated (dog)	4.2	
12	T-1824	Citrated (human)	3.6	
13	T-1824	Citrated (human)	4.2	

* Batch no. 5864, National Aniline and Chemical Company.

† From Drs. J. L. Hartwell and L. F. Fieser.

sodium is trapped between the cells and this presumably represents plasma remaining in the cell residue.

There is as yet no conclusive evidence that this intercellular fluid can be squeezed out completely by centrifugation at higher speeds. The hematocrit reading, to be sure, falls off considerably when the speed is increased (Millar, 1925), but Ponder and Saslow (1930) have suggested that this decrease in the hematocrit may also in part be the result of an actual compression of the cells under the large centrifugal force. Since red blood cells are presumably non-conducting (Fricke, 1924), the amount of plasma trapped in the cell residue can be estimated from the conductivity of the

packed cells. According to numerous reports in the literature (Bugarszky and Tangl, 1892; Fricke and Curtis, 1935; and many others), the measurements show that the residue contains from 2 to 5 per cent of the total plasma, the higher values being obtained when the blood is centrifuged at approximately the same speed and for the same time as that used in the recovery experiments presented in table 1. It is therefore probable that the fraction of the dye lost in these experiments simply represents the proportion of the plasma or serum which remained in the intercellular spaces.[4]

Further support for this conclusion was obtained in experiments 7 and 12 by washing the cell residue with isotonic saline or dye-free plasma. In both cases, the average amount of dye recovered (3.9 per cent) was almost exactly equal to the quantity missed in the original test. The objection might be raised that recovery of the dye by washing the cells does not exclude the possibility that the dye may have been inside the cells. In answer it may be pointed out that if the dye were inside the cells in a free and diffusible form, one would expect on the basis of the fluid content of the cells to find not 4 but 30 per cent present in the cell residue. If, on the other hand, the dye were used up in staining the cell envelope, or in some way combined with the cell constituents, it is unlikely that a single washing would cause it to be liberated.

Recovery of T-1824 from blood after coagulation. The facts presented above in connection with determinations of relative cell volume have an important bearing on the method of collecting blood samples for the analysis of dye in the circulating plasma. If the samples are treated with an anticoagulant such as isotonic oxalate (Hooper, Smith, Belt and Whipple, 1920), which requires a correction for plasma dilution, the relative plasma volume must of course be determined accurately to obtain the true plasma-dye concentration. Since the work of Ponder and Saslow (1930) and others casts considerable doubt upon the reliability of the hematocrit method, it would be desirable either to determine the relative plasma volume by some other means or to eliminate the dilution factor altogether by using an anticoagulant such as heparin which can be added in dry form without causing changes in cell volume. Heparin, unfortunately, colors the plasma sufficiently to interfere with the dye determinations. Another way of avoiding corrections for dilution is obviously to employ serum samples, but most investigators have apparently shunned the serum technic because they suspected that dye might be withdrawn from solution by the blood clot. Indeed, this has been reported in the

[4] It has been suggested by Hirota (1925) that the true relative cell volume may be calculated from the rate of packing as centrifugation proceeds. This method was tried in experiment 7 (table 1), but the calculations based on the corrected hematocrit values still showed a loss of 1.9 per cent of the dye.

case of one vital dye (Hooper et al., loc. cit.), but it will be seen that T-1824 escapes a similar fate regardless of whether the clot is formed from plasma or whole blood.

To test the effect of clotting of plasma a sufficient amount of T-1824 was added to citrated or oxalated plasma to yield a convenient color strength for spectrophotometric readings. The dye solutions were either prepared in triplicate or the original sample was divided into three equal portions. Their optical densities were determined against dye-free plasma obtained from the same blood and diluted to the same extent with saline, thereby cancelling the absorption due to the solvent. Coagulation was produced by heating the samples in a water bath at 56°C. for thirty minutes or by adding a few milligrams of dry $CaCl_2$. The clotted solutions were then

TABLE 2

Effect of clotting in plasma (dog) on dye concentration

EXPERIMENT NUMBER	MEANS OF CLOTTING	OPTICAL DENSITY OF T-1824 AT 620 mμ		DIFFERENCE	ANTI-COAGULANT
		Before	After		
	heat			*per cent*	
1	56°C. for 30′	1.267	1.256	−0.9	Oxalate
2	56°C. for 30′	1.260	1.259	−0.1	Oxalate
3	56°C. for 30′	1.257	1.259	+0.2	Citrate
4	56°C. for 30′	1.237	1.239	+0.2	Citrate
5	Dry $CaCl_2$	1.257	1.255	+0.2	Citrate
6	Dry $CaCl_2$	1.237	1.240	+0.2	Citrate
Control experiments: Effect of adding dry $CaCl_2$ to dye serum containing sodium citrate					
7	Dry $CaCl_2$	1.257	1.258	+0.1	
8	Dry $CaCl_2$	1.234	1.238	+0.3	

centrifuged at high speed for a few minutes to obtain a supernatant fluid free from the coagulum, and the optical densities of the dye solutions determined as before against dye-free plasma treated in exactly the same way. The results are listed in table 2, each value being the average of triplicate determinations.

These experiments show that coagulation of oxalated or citrated plasma by heating produces no demonstrable change in the dye concentration. Furthermore, it was observed that the coagulated material collected at the bottom of each tube by centrifugation was only faintly colored. Coagulation of citrated plasma by addition of a small amount of dry $CaCl_2$ likewise has no measurable effect upon the concentration of T-1824 in solution. That $CaCl_2$ itself does not alter the color of the dye is shown in the two control experiments in which this salt was added to dye serum

containing also sodium citrate. With *oxalated* dye plasma, the results from adding $CaCl_2$ were hopelessly inconsistent. A variable amount of the insoluble calcium oxalate remained suspended in solution, interfering with spectrophotometric determinations of the dye. Centrifugation at 4000 r.p.m. did not bring down this fine precipitate. Moreover, the clot appeared to be highly stained with T-1824.

Similar experiments on whole blood were carried out with citrated samples in which coagulation was initiated by the addition of dry $CaCl_2$. Since dry $CaCl_2$ also causes shrinkage of the erythrocytes, it will in effect dilute the dye solution and in order to make a fair comparison of the dye concentration in the plasma and serum from the same blood it was

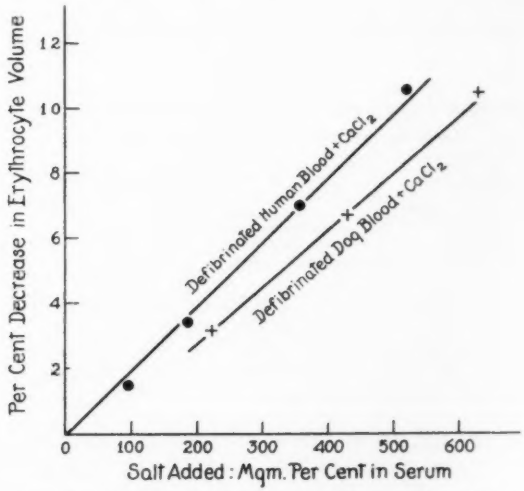


Fig. 1. Showing the changes in relative cell volume caused by the addition of calcium chloride to defibrinated blood.

necessary to determine the extent of this cell shrinkage. Figure 1 shows the changes in relative erythrocyte volume of dog and human blood (defibrinated) when various amounts of $CaCl_2$ are added. On the basis of these data the dye determinations made prior to coagulation were corrected for the dilution caused by the addition of $CaCl_2$. It may be seen from the figures presented in table 3, which includes the results (triplicate determinations) of three experiments on dog blood and one on human blood, that the optical densities of T-1824 in the citrated plasma samples, when corrected for cell shrinkage by $CaCl_2$, were identical with the determinations on the sera obtained from the same dye-tinged bloods.

The possibility of absorption of T-1824 by the blood clot has also been

tested on several occasions by comparing the determinations on serum and oxalated plasma samples collected simultaneously from dogs or human subjects, after injection of the dye. To make the test valid, it is of course necessary to use an oxalate solution that is strictly isotonic with the blood.[5] Also, the oxalate dilution factor must be the same for all plasma samples if the plasma absorption is to be cancelled directly by reading the dye samples against the control. With these conditions satisfied, the agreement between the serum and oxalated plasma values (corrected for dilution by oxalate) is quite good, as may be seen from the results shown in table 4. It is evident from this and the preceding experiments that T-1824 is not withdrawn from solution when the blood samples are permitted to clot. The concentration of this dye in the circulating plasma can therefore be determined on serum samples.

TABLE 3

Effect of clotting of citrated whole blood (dog and human) on dye concentration

Clotting initiated by addition of dry $CaCl_2$

EXPERIMENT NUMBER	BLOOD	OPTICAL DENSITY OF T-1824 AT 620 mμ		DIFFERENCE
		In plasma (corrected for cell shrinkage)	In serum	
				per cent
1	Dog	1.372	1.378	+0.4
2	Dog	1.338	1.340	−0.1
3	Dog	1.189	1.188	−0.1
4	Human	1.310	1.308	−0.1

It should be pointed out that the use of the serum instead of the oxalated plasma technic (Hooper, Smith, Belt and Whipple, 1920) greatly simplifies the plasma-dye method and also increases its accuracy. The tedious hematocrit determinations are of course no longer required. This eliminates the systematic errors due to incomplete separation of plasma and cells by centrifugation and also the uncertainty as to whether or not the anticoagulant is exactly isotonic with the blood on which it is used. Moreover, with serum samples, the dye concentration can be obtained directly by reading the unknown against the control sample collected before the injection of dye. With oxalated plasma samples, direct cancellation of the plasma absorption is possible only if by chance the proportion of oxalate to plasma is the same in both the control and the unknown. For obvious reasons, uniform dilution with oxalate is sometimes difficult

[5] By comparison with hematocrit values obtained on heparinized blood, it has been found that 1.5 per cent potassium oxalate has no effect on the cell volume (Gregersen, Gibson and Stead, to be published).

or even impossible to achieve, especially when the erythrocyte count of the circulating blood is fluctuating. There is a further advantage in avoiding the dilution of the samples with oxalate since this dilution calls for the injection of a larger quantity of dye in order to establish a concentration that can be determined accurately. This is of practical importance in repeated experiments on human subjects where it is desirable to reduce the dose as much as possible to prevent staining.

TABLE 4

Splenectomized dog

Body weight 26.7 kgm. A series of simultaneous determinations of T-1824 in serum and oxalated plasma obtained from the same samples of blood taken in succession after the injection of dye. For oxalated plasma, 4 parts of whole blood were mixed with 1 part of 1.5 per cent potassium oxalate (isotonic) and the samples centrifuged at 3000 r.p.m. for 30 minutes (radius 15 cm.). For serum the blood was allowed to clot under oil.

TIME	VEIN	OPTICAL DENSITY OF T-1824 (620 mμ) IN THE CIRCULATING PLASMA			
		From serum	From plasma (corrected for dilution)	Oxalate correction factor	Per cent cells
a.m.					
11:25	Right jugular (dye-free sample)			0.689	43.4
11:26	Right jugular 3 cc. 1 per cent T-1824*				
11:30	Left jugular	0.937	0.918	0.685	44.8
11:32	Left jugular	0.922	0.915	0.680	45.3
11:36	Left jugular	0.887	0.888	0.686	43.5
11:41	Left jugular	0.871	0.858	0.689	43.2
11:51	Left jugular	0.854	0.859	0.689	43.7
Average		0.894	0.888		

* From Meister, Lucius and Brünning. For the spectral absorption curve of this sample of T-1824 see Gregersen and Gibson (1937) figure 8, curve 1.

SUMMARY AND CONCLUSIONS

1. When a known amount of brilliant vital red or T-1824 is mixed with whole blood in vitro, about 4 per cent of the dye fails to appear in the plasma separated by centrifugation at 3000 r.p.m. (radius 15 cm.) for 30 minutes (table 1).

2. Evidence is presented which shows that this fraction of the dye is merely trapped in the cell residue with a proportionate amount of plasma and not taken up by the red blood cells. Insofar then as their reaction with red blood cells is concerned, T-1824 and brilliant vital red are satisfactory for determinations of plasma volume.

3. It has been shown that coagulation of either plasma or whole blood containing T-1824 does not reduce the amount of the dye in solution (tables 2, 3 and 4). A truer value for the concentration of T-1824 in the circulating plasma may therefore be obtained by using the serum rather than the oxalated plasma technic (Hooper, Smith, Belt and Whipple, 1920) which is subject to the uncertainties inherent in the hematocrit determinations that it entails.

REFERENCES

BLEIBTREU, M. AND L. BLEIBTREU. Pflüger's Arch. **51:** 151, 1892.

BUGARSZKY, S. AND F. TANGL. Centralbl. f. Physiol. **11:** 297, 1892.

ERLANGER, J. Physiol. Rev. **1:** 177, 1921.

FRICKE, H. J. Gen. Physiol. **6:** 375, 1924.

FRICKE, H. AND H. J. CURTIS. J. Gen. Physiol. **18:** 821, 1935.

GREGERSEN, M. I. J. Lab. and Clin. Med. 1937. (In press.)

GREGERSEN, M. I. AND J. G. GIBSON. This Journal **120:** 494, 1937.

GREGERSEN, M. I., J. G. GIBSON AND E. A. STEAD. This Journal **113:** 54, 1935.

HARRIS, D. T. Brit. J. Exper. Path. **1:** 142, 1920.

HARTWELL, J. L. AND L. F. FIESER. Organic syntheses **16:** 12, 1936.

HIROTA, K. J. Biophysics **1:** 233, 1925.

HOOPER, C. W., H. P. SMITH, A. E. BELT AND G. H. WHIPPLE. This Journal **51:** 205, 1920.

JENKINS, C. E. Brit. J. Exper. Path. **13:** 127, 1932.

KEITH, N. M., L. G. ROWNTREE AND J. T. GERAGHTY. Arch. Int. Med. **16:** 547, 1915.

LINDHARD, J. This Journal **76:** 497, 1926.

MILLAR, W. G. Quart. J. Exper. Physiol. **15:** 187, 1925.

OBERST, F. W. J. Biol. Chem. **108:** 153, 1935.

PONDER, E. AND G. SASLOW. J. Physiol. **70:** 18, 1930.

SEYDERHELM, R. AND W. LAMPE. Ztschr. f. d. ges. Exper. Med. **30:** 403, 1922.

STEWART, G. N. J. Physiol. **24:** 356, 1899.

FACTORS INFLUENCING THE CONCENTRATIONS OF THE SERUM PROTEIN, CHLORIDE AND TOTAL FIXED BASE OF THE DOG DURING EXERCISE[1, 2]

FREDERIC W. SCHLUTZ AND MINERVA MORSE[3]

From the Department of Pediatrics of the University of Chicago

Received for publication August 2, 1937

The results to be presented in this paper represent one phase of a problem undertaken for the purpose of making a complete study of changes which occur in the acid-base system of the serum of the dog as a result of strenuous, long-continued exercise. Serum protein, chloride and total fixed base were at first determined routinely, in hopes that the results would help to explain the fall in bicarbonate concentration which invariably occurs as a result of such exercise. As data accumulated, however, it became apparent that the correct interpretation of the changes in serum protein, chloride and total fixed base which were observed would furnish considerable information concerning the events occurring in the dog's organism during exercise.

EXPERIMENTAL PROCEDURE. Ten short-haired male dogs weighing 9 to 14 kilograms were used in the experiments. Exercise consisted either in running on the motor-driven treadmill or swimming in a tank of water maintained at 38°C. Conditions for treadmill exercise were varied by raising the incline of the treadmill from 15 to 22½ per cent or by increasing the rate of running from 5 to 6 miles per hour. The room temperature ranged usually from 20 to 22°C.; in a few instances from 26 to 30°C. In a few cases water was allowed at intervals during the running, but in the majority of experiments it was withheld until a 2 hour recovery sample had been taken. In the swimming experiments, work was made more difficult by hanging a 300 to 500 gram weight from the dog's neck. In

[1] This work has been conducted under a grant from the Douglas Smith Foundation at the University of Chicago.

[2] Reports of the work discussed in this paper have been presented at the meetings of the American Society of Biological Chemists at Detroit in March, 1935, at Washington D. C. in March, 1936, and at Memphis, in April, 1937. (Proc. Am. Soc. Biol. Chem. **8:** lxix (1935); J. Biol. Chem. **109:** (1935). Proc. Am. Soc. Biol. Chem. **8:** lxxiv (1936); J. Biol. Chem. **114:** (1936). Proc. Am. Soc. Biol. Chem. **8:** lxxi (1937); J. Biol. Chem. **119:** (1937).

[3] The authors are indebted to Miss Helen Oldham and Miss Elizabeth Riddle for assistance in the experimental work. They wish to express to Prof. A. Baird Hastings their deep appreciation of his valuable criticism and suggestions.

two experiments, isotonic sodium chloride solution was substituted for tap water in the swimming tank. In all cases, the dogs were trained for several weeks before the experiments were performed. Exercise was continued to exhaustion. The duration of the exercise varied, depending upon the individual dog and the conditions, from 2 to 12 hours for swimming, and from 1 to 6 hours for treadmill exercise. In general the time to exhaustion for a given dog was shortened by increasing the degree of incline, the rate of running, or the room temperature. In every case, the work performed represented that due to strenuous, long-continued exercise as opposed to short, violent exercise.

In the treadmill experiments, the dogs were weighed before exercise was begun and after the termination of the exercise. The weight was corrected for the blood drawn during the experiment.

Three venous blood samples, 20 to 25 cc. each, were drawn under oil during the course of the experiment; the first with the dog in the resting state before exercise; the second immediately after exercise ceased (1 to 3 minutes were usually required for the drawing of the sample); and the third after two hours of rest following exercise.

In a few experiments the urine was analyzed before and after exercise. For this purpose the dogs were kept in metabolism cages. Twenty-four hour specimens of urine were collected for the three days preceding exercise and for two days following. The urine was collected in bottles containing oil and kept at a temperature of 15°C. or below. The pH and CO_2 content were determined in specimens not more than twenty-four hours old. The pH was determined electrometrically with a quinhydrone electrode, and the CO_2 content in the Van Slyke manometric apparatus (1). While no claim is made for the accuracy of the pH and CO_2 content as determined, since the samples were not collected directly under oil, it is believed nevertheless that any large differences due to exercise could be detected. Determinations of inorganic phosphate, chloride and total base were also made.

In order to determine cell volume, a small amount of each sample was transferred to a tube containing powdered potassium oxalate, from which it was later transferred to capillary hematocrit tubes and centrifuged at 3000 revolutions per minute for 20 minutes.

The remainder of the sample was transferred under oil to 50 cc. centrifuge tubes and centrifuged at once. The clot which formed with the serum was broken up by pressing against the wall with glass rods and the sample centrifuged again. The serum was pipetted off under oil. In a few experiments the cells were also analyzed. In such cases the residual cells were centrifuged a second or third time, the last traces of serum removed as completely as possible and the samples of cells for analysis drawn up from the bottom of the centrifuge tube by pipette.

Water content was determined by drying 1 cc. samples to constant weight in an oven at 105°C., serum pH by the colorimetric method of Hastings and Sendroy (2), chloride by the wet ashing method of Laudet (3) using concentrated nitric acid and saturated potassium permanganate solution.

TABLE 1

Average concentration of serum protein, chloride and total fixed base before and after exercise under various conditions

CONDITIONS	DOG NUMBER	NUMBER OF EXPERIMENTS	TIME TO EXHAUSTION	PROTEIN		CHLORIDE		TOTAL FIXED BASE	
				Initial	Exhaustion	Initial	Exhaustion	Initial	Exhaustion
			minutes	*grams per kgm. H_2O*	*grams per kgm. H_2O*	*m.-eq. per kgm. H_2O*	*m.-eq. per kgm. H_2O*	*m.-eq. per kgm. H_2O*	*m.-eq. per kgm. H_2O*
Treadmill. H_2O withheld	1	8	67	70.3	72.8	117.0	121.1	170.5	174.9
	2	4	68	67.5	77.7	116.1	123.6	169.8	177.5
	3	3	83	63.5	64.6	121.1	128.9	168.2	171.5
	2	3	114	69.3	73.5	113.7	125.2	169.3	179.4
	4	5	118	70.3	88.1	115.9	128.2	170.5	183.4
	5	5	132	66.8	67.3	118.7	126.5	168.0	175.2
	6	5	133	63.9	67.5	117.6	128.4	169.1	179.0
	4	5	149	70.3	88.1	115.9	128.2	170.5	183.4
	2	4	166	66.5	68.4	115.2	124.8	168.0	177.7
	5	5	304	67.1	71.1	118.2	133.7	167.0	180.6
Treadmill. H_2O offered at intervals	2	7	101	66.8	67.6	116.4	118.6	170.2	171.3
	7	6	122	79.3	80.7	123.2	120.9	174.6	170.3
	5	3	295	65.5	62.0	119.5	119.5	168.6	167.7
	4	2	348	67.4	70.6	116.5	123.5	165.7	170.3
Swimming at 38°C.	2	6	126	67.3	62.5	117.0	98.4	168.8	147.8
	4	2	139	72.0	63.1	117.8	97.7	172.5	148.3
	8	6	272	74.8	65.2	122.1	102.8	172.7	154.3
	2	3	485	75.1	65.7	116.0	90.6	165.0	137.8
Swimming in isotonic NaCl solution	2	2	110	66.3	53.5	117.1	124.2	174.2	171.0

Total fixed base was determined at first by the method of Stadie and Ross (4), carrying out the digestion with sulphuric and nitric acids in Pyrex test tubes, as recommended by Fiske (5). In later determinations, the method of Fiske was adopted, since it gave slightly higher values which were more consistent with the sodium values found in the same sample, and since it also gave more uniform resting values. Phosphate was removed in both methods.

In earlier experiments protein was determined as the sum of albumin and globulin by the colorimetric method of Greenberg (6). In later experiments Kjeldahl nitrogen was determined by the micro technic of Pregl (7) and protein calculated by multiplying the nitrogen value (after correcting for non-protein nitrogen) by 6.25. There was no essential difference in the results by the two methods.

Discussion of results. In most of the early experiments serum protein, chloride and total fixed base have been determined only on the initial and exhaustion samples. Table 1 gives the average results of such determinations for the different dogs when exercised under various conditions.

The concentrations of protein, chloride and total fixed base increase as a result of treadmill exercise, decrease as the result of swimming. If the dog is allowed to drink water while running, the rise in protein, chloride and total fixed base is often prevented. These results suggest that the concentrations of serum protein, chloride and total fixed base are dependent upon the water content of the body. During treadmill exercise water may be lost from the body as part of the heat-dissipating mechanism, and the concentrations of serum protein, chloride and fixed base may rise. During swimming, the concentrations of serum protein, chloride and fixed base may fall as a result of the water swallowed.

That the dog does swallow water during swimming is shown by the results observed when isotonic salt solution is substituted for tap water in the swimming tank. The concentration of serum protein falls as in the other experiments, but the concentration of chloride increases and that of base falls but slightly.

Figure 1 shows that loss of body weight occurs during treadmill exercise and that the loss of weight per kilo of body weight is roughly related to the duration of the exercise period. The parabolic form of the regression curve indicates that a relatively larger loss of weight occurs per unit of time in the shorter exercise periods. This would appear to be significant since the shorter periods are characteristic either of poor runners or of experiments in which a greater output of work is demanded as a result of the increased speed or increased incline of the treadmill, or in which heat-dissipation is made more difficult by a higher room temperature.

Figure 2 shows the results of plotting the changes in serum protein, fixed base and chloride against loss in body weight. Of these, the rise in serum chloride appears to be very closely related to loss in body weight; the rise in serum base shows a poorer correlation, while serum protein concentration shows only a general tendency to increase as the body loses weight. Since loss of body weight represents largely a loss of body water, the close correlation between gain in serum chloride concentration and

loss of body weight suggests that serum chloride concentration during exercise is determined to a great extent, if not entirely, by the water content of the body. The greater scattering of points in the case of fixed base may be due to the greater experimental error involved in this determination. Serum protein is most probably governed by other factors as well as by the water content of the body.

Figure 3 shows clearly that during the exercise the concentrations of serum protein, chloride and fixed base are not governed entirely by one

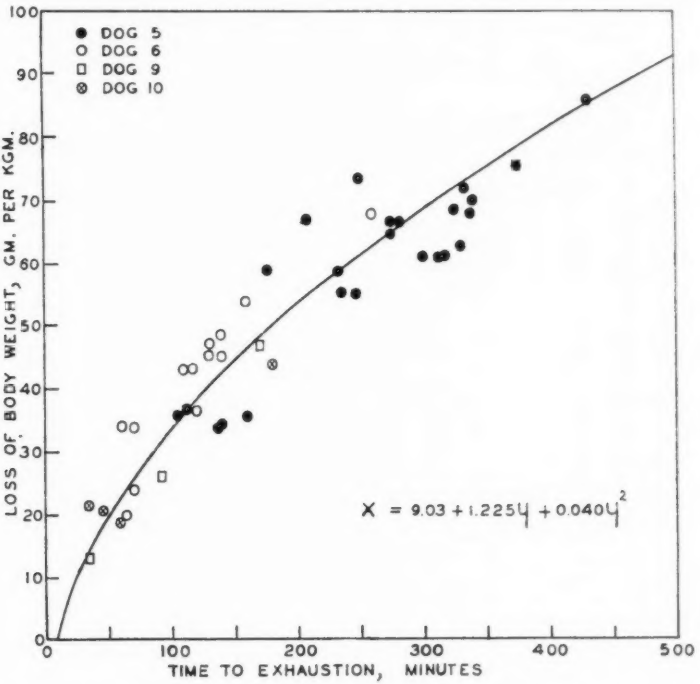


Fig. 1. Relationship between loss of body weight and the time to exhaustion

and the same factor, and that factors other than loss of water from the system must be considered, especially in the case of serum protein. In this diagram average exhaustion concentrations are represented graphically as percentages of the corresponding initial concentrations. The increase of serum protein is much larger in the case of dog 4, while in the case of dog 5 it is noticeably lower than the corresponding base and chloride concentrations. In the swimming experiments, the decrease in serum protein concentration is not as great as that of chloride and base.

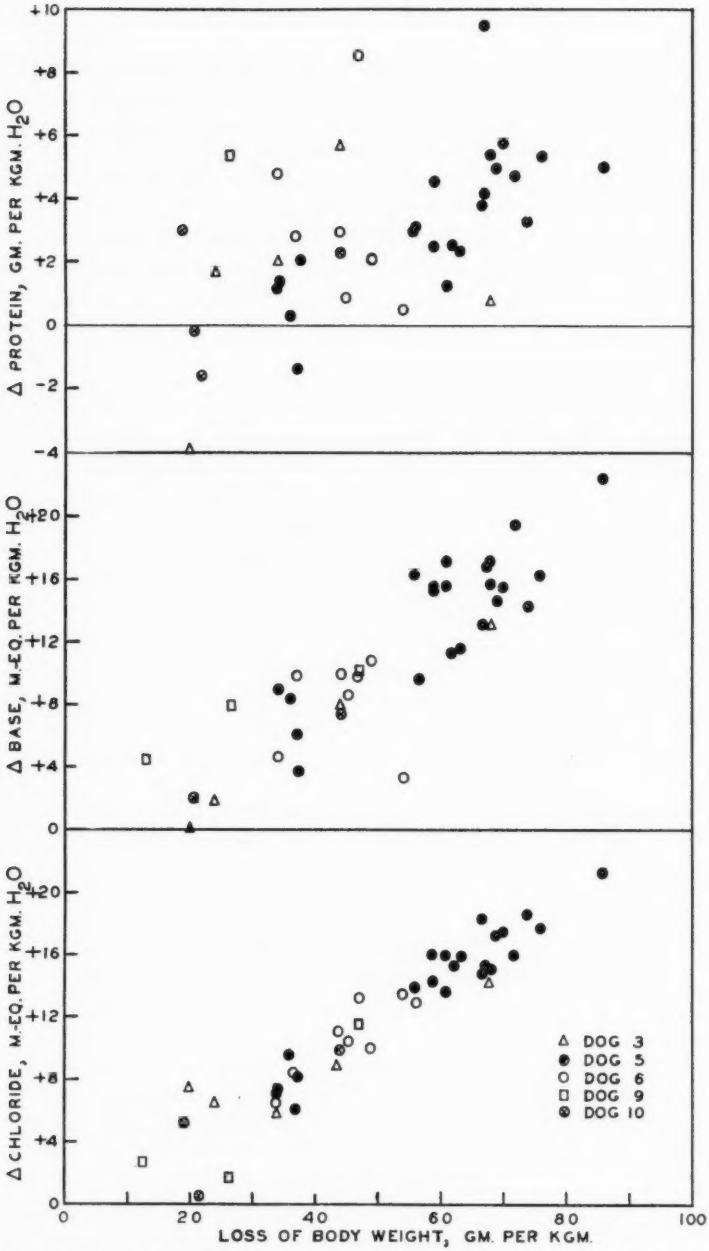


Fig. 2. Relation of serum chloride, fixed base and protein to loss of body weight as the result of exercise.

The difference in response of serum protein to exercise as compared with that of chloride and fixed base is illustrated also in table 2. This table gives the results of individual experiments in which the concentrations of serum protein, chloride and fixed base were determined in the two-hour recovery sample as well as in the initial and exhaustion samples. With few exceptions concentrations of serum chloride and base change very little from the exhaustion concentrations during the two-hour rest

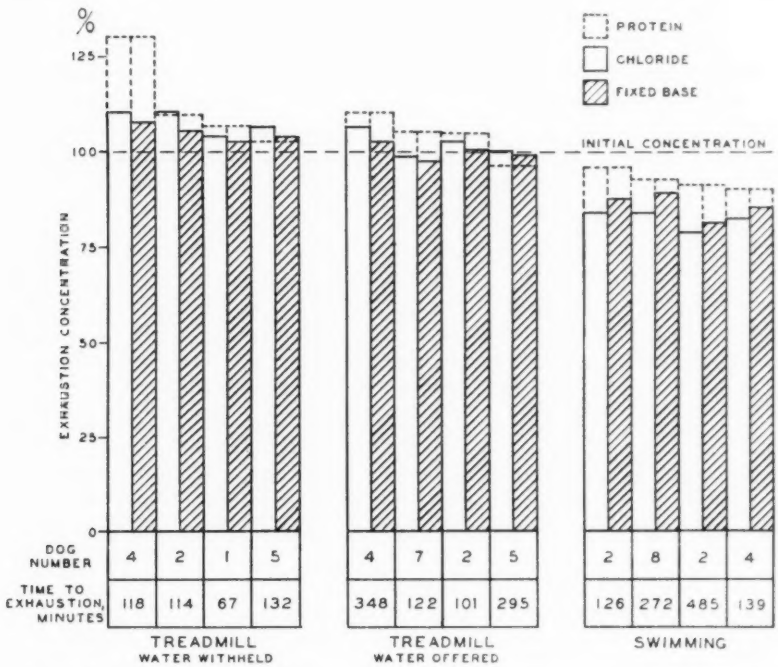


Fig. 3. Effect of treadmill exercise upon the concentrations of serum protein, chloride and total fixed base. Exhaustion concentrations are expressed as per cent of the initial concentrations.

period, while the concentration of serum protein often returns approximately to its initial value.

Consideration of the character of the capillary membrane suggests that altered hydrostatic or osmotic forces are responsible for much of the protein changes during exercise and recovery. The capillary membrane is freely permeable to diffusible ions such as sodium and chloride, but Keys and Taylor (8) have shown that not more than 1 per cent of the serum protein can pass through the capillary walls during exercise.

With increase in hydrostatic pressure within the blood system, or of osmotic pressure within the tissue cells, fluid containing fixed base and

TABLE 2

pH and serum protein, chloride and total fixed base before and after treadmill exercise and after a recovery period of two hours

DOG NUMBER	EXPERIMENT NUMBER	TIME TO EXHAUSTION	pH			PROTEIN			CHLORIDE			TOTAL FIXED BASE		
			Initial	Exhaustion	Recovery	Initial	Exhaustion	Recovery	Initial	Exhaustion	Recovery	Initial	Exhaustion	Recovery
		minutes				*grams per kgm. serum H_2O*			*m.-eq. per kgm. serum H_2O*			*m.-eq. per kgm. serum H_2O*		
3	3	64	7.34	7.54	7.38	66.7	62.8	60.6	119.4	126.9	125.4	170.6	170.4	177.2
	4	70	7.38	7.47	7.36	60.3	62.0	59.3	121.2	127.7	124.6	168.0	170.2	171.6
	5	261	7.38	7.42	7.23	62.1	62.9	62.4	118.1	132.5	131.6	169.5	182.6	175.2
	7	115	7.37	7.55	7.37	63.4	69.1	64.8	122.7	132.0	131.7	166.0	174.0	175.1
Mean...		128	7.37	7.49	7.34	63.1	64.2	61.8	120.4	129.8	128.3	168.5	174.3	174.8
6	3	158	7.43	7.49	7.39	64.1	70.1	63.6	116.0	129.5	132.4	167.0	176.0	178.6
	4	110	7.40	7.57	7.40	59.4	62.4	58.7	117.8	129.0	130.0	169.0	179.1	178.9
	6	142	7.42	7.52	7.39	63.5	63.6	62.4	117.9	128.1	128.0	169.6	180.4	178.0
	12	118	7.40	7.49	7.39	64.8	67.6	65.1	118.7	127.0	128.0	168.6	178.4	178.3
	13	138	7.41	7.54	7.40	68.0	73.7	68.9	117.6	128.3	128.2	171.4	181.2	179.9
Mean...		133	7.41	7.52	7.39	63.9	67.5	63.7	117.6	128.4	129.3	169.1	179.0	178.7
5	26	112	7.42	7.43	7.38	65.1	67.2	68.2	118.2	124.4	123.5	165.2	169.0	172.3
	27	163	7.37	7.34	7.36	69.5	69.8	70.7	119.8	129.5	129.1	169.5	178.0	173.4
	28	137	7.39	7.39	7.34	66.0	67.2	66.8	120.0	127.0	127.5	171.6	180.4	180.0
	29	140	7.43	7.36	7.41	65.6	66.9	67.6	117.4	125.0	124.3	168.7	177.4	175.7
	30	108	7.41	7.47	7.37	67.9	65.5	65.7	118.2	126.5	126.5	165.0	171.1	173.3
	37	247	7.45	7.40	7.38	67.0	70.1	73.7	119.1	133.0	130.6	168.0	177.5	181.0
	38	281	7.42	7.40	7.35	66.9	71.1	71.5	120.0	135.0	135.9	164.0	180.9	178.3
	40	337	7.39	7.38	7.34	64.8	70.2	72.8	117.3	132.7	132.5	164.0	179.6	179.3
	42	330	7.43	7.39	7.38	68.2	70.5	71.4	116.2	132.2	133.6	167.0	178.5	179.2
	43	325	7.43	7.38	7.34	68.5	73.5	71.5	118.4	135.8	136.6	172.0	186.7	185.1
	45	313	7.39	7.42	7.38	69.5	70.8	69.5	120.6	134.2	133.0	174.5	190.1	188.2
	46	325	7.41	7.34	7.30	66.4	70.2	70.5	118.8	137.3	136.3	173.9	191.0	187.4
	47	333	7.41	7.34	7.34	63.4	66.0	66.4	120.0	136.2	136.6	172.2	191.6	188.9
	48	318	7.36	7.37	7.36	63.3	65.5	67.7	118.4	132.5	132.9	170.7	187.9	186.2
	50	236	7.47	7.42	7.42	69.4	73.4	72.3	120.8	133.8	134.2	177.7	194.2	194.9
	51	277	7.42	7.46	7.41	67.8	70.8	71.0	119.7	137.4	135.9	174.5	191.9	192.1
Mean...		249	7.41	7.39	7.37	66.8	69.3	69.8	118.9	132.0	131.8	169.9	182.9	182.2

chloride may diffuse out through the capillary walls, leaving the serum protein relatively more concentrated. With decrease in hydrostatic or osmotic pressure, such as occurs during recovery, fluid may diffuse into

the blood stream and reduce the protein concentration while the base and chloride concentrations remain practically unchanged. Such an explanation was used by Dill, Talbott and Edwards (9) to interpret the effect of short vigorous exercise in man.

The case of dog 5, an especially good runner, is interesting in this connection. The serum protein concentration increased during exercise, but relatively less than that of chloride and base; it changed very little on recovery, tending to increase rather than decrease. If loss of protein by sampling is taken into consideration, the concentration of protein on recovery agrees with the value calculated on the basis of dehydration corresponding to chloride change. In the case of this dog, therefore, very little change in hydrostatic or osmotic pressure occurred during exercise and recovery, a fact which may account for his capacity for running.

Figure 3 shows that in the treadmill experiments the serum chloride concentration increases relatively more than that of fixed base, while in the swimming experiments its decrease in concentration is greater than that of base. The results of swimming experiments are probably explained by the fact that the vomiting which usually occurs, presumably due to the large quantities of water swallowed, deprives the system of more chloride than base. The relatively greater increase of serum chloride over fixed base in the treadmill experiments results in a decrease in the serum base to chloride ratio, which is not so easily explained. The serum either gains chloride relative to base or loses base relative to chloride. Both of these possibilities have been investigated.

Presumably any gain of chloride by the serum is attained in conformity with the Gibbs-Donnan laws of membrane equilibrium. The serum is separated by membranes from two fluid systems, the red blood cells and the interstitial fluids. It is well known that a rise of serum pH such as often occurs in treadmill exercise as the result of over-ventilation, is associated with a transfer of bicarbonate ions from the serum into the cells and a transfer of chloride and water in the reverse direction. Such a transfer may increase the serum chloride relative to base but its effect would be reversible and when the pH falls during recovery the base to chloride ratio should rise again.

The results of these experiments, however, show no such relationship of pH shift to alterations in the base to chloride ratio. Experiments in which a fall of pH occurs often show a greater decrease in the base to chloride ratio than those in which a considerable rise occurs. In the majority of experiments a fall of pH occurs during recovery, but seldom is there a corresponding decrease in the base to chloride ratio. Any transfer of chloride between cells and serum associated with pH shift is masked by other factors.

The effect of the reservoir of interstititial fluid has also been considered.

With the aid of several assumptions, we have calculated from the cell and serum data of a few experiments the change in the water, chloride and total fixed base content of those fluid phases of the dog's organism which contain appreciable chloride, the red cells, blood serum and other extracellular fluids. For this purpose it is assumed 1, that one kilo of the organism contains initially 44 grams of serum (on the basis that one-thirteenth of body weight represents total blood weight) and 226 grams of extracellular fluid (exclusive of the blood system) (10); 2, that the capillary and cell walls are impermeable to protein and therefore change in protein concentration may be used as a measure of change in serum water content; 3, that the walls of both the red cells and tissue cells are practically impermeable to fixed base and the change in the concentration of base in the red cells may be used as a measure of the change in their water content; 4, that during the experiment protein and chloride were not lost except by sampling (correction has been made for such loss); and 5, that the concentration of fixed base and chloride in the extracellular fluid are governed by the equation:

$$\frac{[Cl]_s}{[Cl]_f} = \frac{[B]_f}{[B]_s} = 0.95$$

The results of such calculations in two typical experiments are shown in figure 4, the data for which are given in table 3. Dog 5 is a very good runner, dog 6 a moderately good runner. The serum of dog 6 shows a marked rise in pH and in protein concentration as the result of exercise, and an approximate return to the initial concentration after 2 hours of recovery. The serum of dog 5 shows only a slight rise in pH and in protein concentration, coincident with a relatively higher chloride and fixed base concentration, and little change during the recovery period. The calculated contents of the three fluid phases, red cells, serum and extracellular fluid, as shown in the chart, are expressed as areas relative to an initial kilo of serum water. The chart shows: 1, a loss of water from the total fluid system in both cases; 2, a difference in water content of the serum and extracellular fluid in the two dogs at exhaustion attributed to the higher hydrostatic pressure of the blood system or to the greater osmotic pressure of the tissue cells of dog 6; 3, a shift of chloride between cells and serum in response to pH shift; 4, and a loss of fixed base from the total fluid system. The actual amount of fixed base lost from the system may not be represented accurately because a 1 per cent error in the determination of serum fixed base is magnified over five times when it is transferred to the larger volume of extracellular fluid. It would seem of some significance, however, that a loss of base was observed in each of 7 similar experiments.

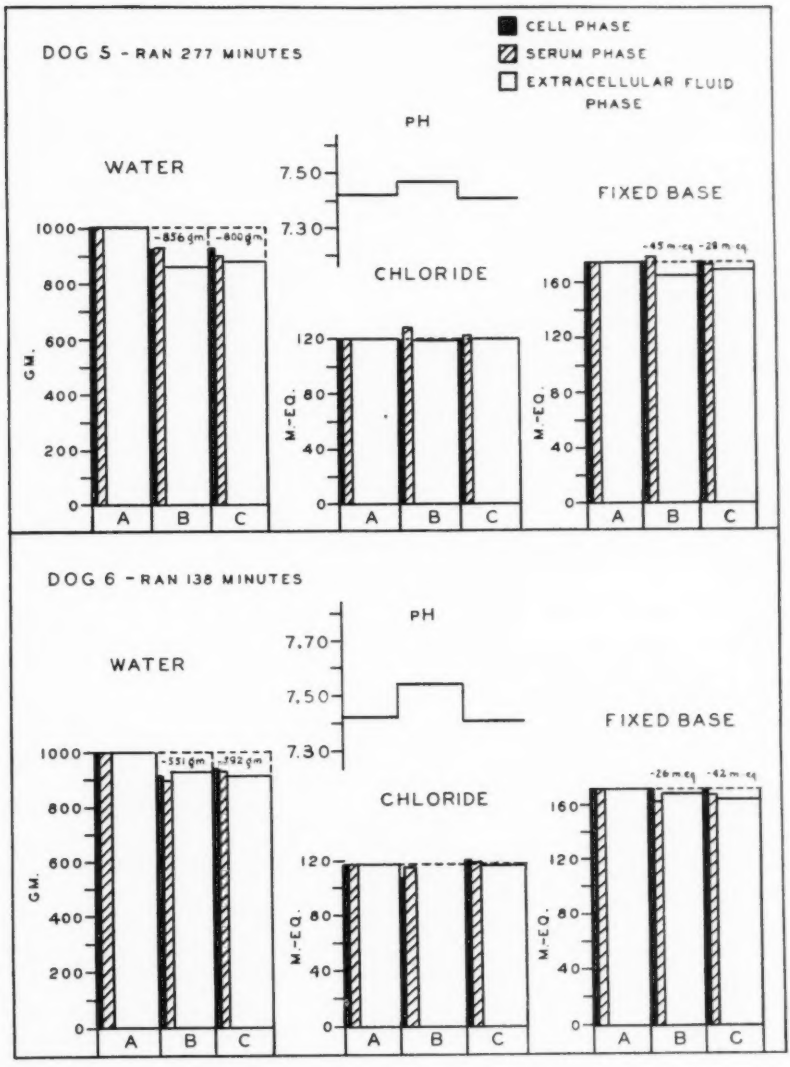


Fig. 4. The effect of exercise upon the water, base and chloride contents of the red cell, serum and extracellular fluid phases of the dog's organism. The areas represented are relative to an initial kilo of serum water. *A* represents the initial state, *B* the exhaustion state and *C*, that after 2 hours of recovery.

TABLE 3

Changes in serum and cells as the result of exercise and recovery

DETERMINATION	DOG 5			DOG 6		
	Initial	Exhaustion	Recovery	Initial	Exhaustion	Recovery
Time to exhaustion, minutes		277			138	
Body weight, kgm.	10.84	10.11		11.13	10.60	
Rectal temperature, °C.	39.5	39.7	39.5	38.7	40.5	38.7
Cell volume, per cent	47.8	50.4	48.6	47.4	45.8	41.2
Serum pH	7.42	7.46	7.41	7.41	7.54	7.40
Serum total solids, grams per liter serum	79.2	81.4	83.7	80.0	86.2	82.0
Serum H_2O, grams per liter serum	941.6	935.8	937.3	939.8	933.4	937.2
Serum protein, grams per kilogram H_2O	67.8	70.8	71.0	68.0	73.7	68.9
Serum chloride, m.-eq. per kilogram H_2O	119.7	137.4	135.9	117.6	128.3	128.2
Serum fixed base, m.-eq. per kilogram H_2O	174.5	191.9	192.1	171.4	181.2	179.9
Cell total solids, grams per liter cells	357.3	378.5	374.9	344.3	366.8	358.6
Cell H_2O, grams per liter cells	682.9	677.8	682.2	705.2	684.6	688.8
Cell chloride, m.-eq. per kilogram H_2O	94.2	100.6	103.8	88.0	87.0	96.4
Cell fixed base, m.-eq. per kilogram H_2O	172.8	186.9	192.9	167.9	183.7	179.1

TABLE 4

Data used in calculating the per cent of body weight loss due to loss of water from the red cell, serum and extracellular fluid phases of the dog's organism

DOG NUMBER	EXPERIMENT NUMBER	CALCULATED LOSS OF H_2O FROM CELL-SERUM-EXTRACELLULAR FLUID PHASES (H_2O)	LOSS OF BODY WEIGHT (Δ BODY WEIGHT)	$\frac{H_2O}{\Delta \text{ BODY WEIGHT}} \times 100$
		grams	*grams*	*per cent*
6	12	207	409	50.7
	13	247	505	48.9
5	46	405	717	56.5
	47	343	747	45.7
	48	311	628	49.5
	50	293	595	49.3
	51	350	705	49.6
Mean		308	615	50.0

In table 4 it is shown that the loss of water from the red cell-serum-extracellular fluid system during exercise represents about 50 per cent of the total loss of body weight. Loss of carbon as a result of metabolism and loss of water from intracellular fluids will account for the balance. Since the tissue cells are freely permeable to water, and since presumably the osmotic pressure of all the cells and fluids of the body is the same,

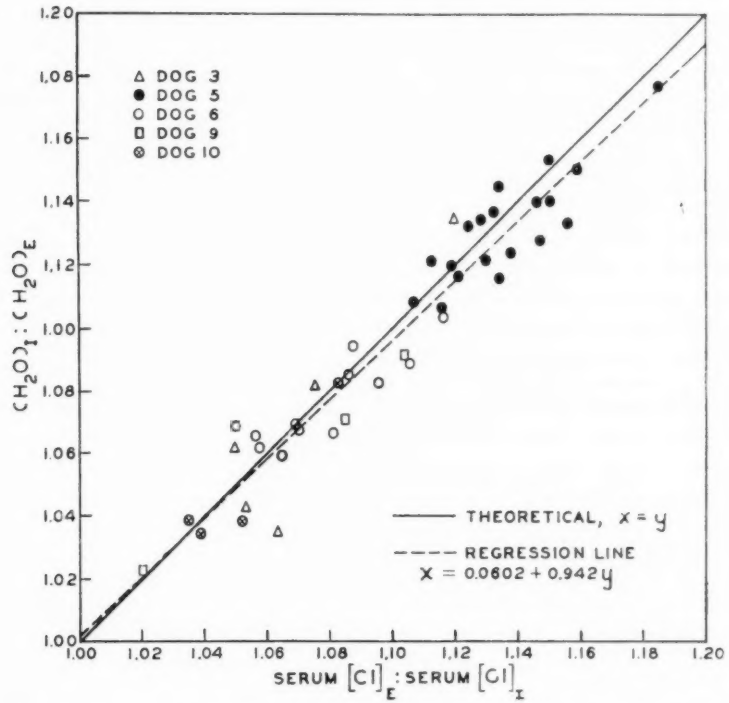


Fig. 5. To show that changes in serum chloride concentration during exercise are due to loss of water from the red cell-serum-extracellular fluid system. (H_2O) represents the water content of the red cell-serum-extracellular fluid system of the dog. *I* and *E* refer to the initial and exhaustion states respectively.

the relatively smaller loss of water from the intracellular fluids would seem to indicate that the osmotic pressure of the non-diffusible ions of the tissue cells has materially increased as the result of exercise.

Figure 5 shows that the change in serum chloride during exercise is probably determined by the water content of the red cell-serum-extracellular fluid phases of the organism. In the diagram the ratio, $(H_2O)_I:(H_2O)_E$,

is plotted against the ratio Serum $[Cl]_E$:Serum $[Cl]_I$, where I and E refer to the initial and exhaustion states respectively and (H_2O) to the water content of the red cell-serum-extracellular fluid system. The initial water content of the system is calculated as 29.3 per cent of the initial body weight, assuming that 1 kilogram of organism contains 33 grams cells, 44 grams serum and 226 grams extracellular fluid. The water content of the same system at exhaustion, $(H_2O)_E$, is found by subtracting 50 per cent of the loss of body weight from $(H_2O)_I$. The line drawn is the theoretical line upon which the points would fall if the chloride concentration expressed as m.-eq. of chloride per kilogram water varies inversely as the water content of the fluid system. The regression line for the plotted points calculated by the method of least squares is $y = 0.0602 + 0.942\,x$, a line which corresponds almost exactly with the theoretical line. The correlation coefficient, r, is + 0.96.

A consideration of these results indicates that the reduction in the base to chloride ratio of the serum is therefore to be attributed, not to any gain of relatively larger amounts of chloride than of base by the serum, but to loss of fixed base from the total fluid system. Two possible channels for such loss are the salivary glands and the kidneys. Appreciable loss by salivation would seem to be excluded in the case of the dogs used in these experiments. The one sample of saliva it was possible to collect contained fixed base and chloride in approximately the same ratio as in the serum. Dogs who have shown most marked decreases in the base to chloride ratio of serum salivated very little.

On the other hand, while base is not excreted in abnormally large amounts by the kidneys during exercise, there is a retention of chloride, and probably a normal excretion of fixed base with anions other than chloride, a fact which may explain the decreased base to chloride ratio of the serum. The average results of four experiments are shown in figure 6 and table 5. In each experiment urine has been collected for 72 hours before and 48 hours after a 5 hour run on the treadmill.

An increased volume of urine is excreted for the 48 hours following exercise. In spite of the increased urinary excretion there is no increase in the amount of fixed base excreted but a marked decrease in that of chloride. The pH of the urine, not shown on the chart, tends to fall as the result of exercise.

Table 5 shows that the composition of the urine excreted in the 24 hours including and following the exercise period is definitely altered. The post-exercise urine contains a higher percentage of acid phosphate and especially of sulphate, a slightly higher percentage of bicarbonate, but a much lower percentage of chloride. If such urine is excreted during the actual period of exercise, it would serve to explain in large part the loss of fixed base from the red cell-serum-extracellular fluid system.

The effects of over-ventilation were first considered as the possible cause of loss of base with exercise. Our findings bear a marked resemblance to those reported by Peters, Bulger, Eisenman and Lee (11) in a case of encephalitis with prolonged hyperventilation. The blood serum changes, decreased serum bicarbonate and fixed base, and increased

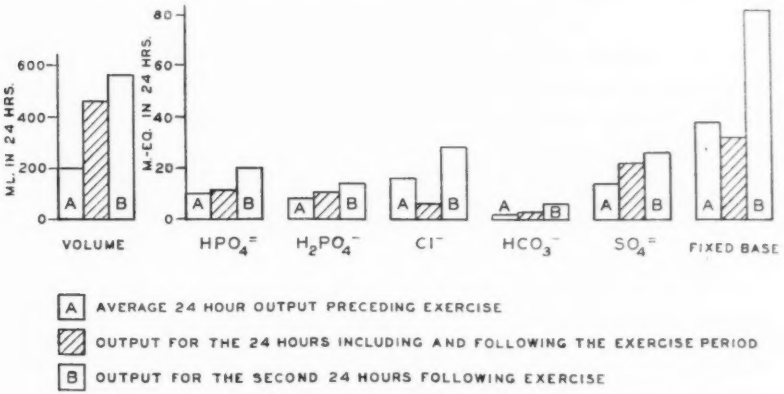


Fig. 6. Effect of exercise upon urine excretion. Average of 4 experiments.

TABLE 5

*The effect of exercise upon the composition of dog's urine**

	BEFORE EXERCISE	FIRST 24 HOURS AFTER EXERCISE	SECOND 24 HOURS AFTER EXERCISE
	per cent	*per cent*	*per cent*
$H_2PO_4^-$	15.1	21.0	14.3
$HPO_4^=(\times 2)$	21.6	20.8	21.5
Cl^-	30.5	11.4	29.5
HCO_3^-	3.5	5.0	6.4
$SO_4^=(\times 2)$	29.3	41.8	28.3
Inorganic anions	100.0	100.0	100.0
Fixed base	81.7	71.5	76.3

* Quantities are expressed as m.-eq. per 100 m.-eq. of total inorganic anions.

chloride, approximate those occurring in the dogs during exercise. Peters (12) suggests that these results may be due to alterations of the relative rates of excretion of chloride and bicarbonate in the urine. The recent report of Briggs (13) of an increased excretion of fixed base by the kidneys following over-ventilation in man has an important bearing on the subject. Different results are obtained with the dogs: the amount of fixed base

excreted was not increased with exercise and the actual amount of base eliminated in combination with bicarbonate was small, approximately 1 m.-eq. Therefore the loss of base which occurred as the result of exercise in the dog is thought to be due not to over-ventilation but to an increased phosphate and sulphate metabolism.

SUMMARY

1. The results of strenuous, long-continued exercise under various conditions of treadmill exercise and swimming upon the protein, chloride and total fixed base concentrations of the blood serum of the dog have been presented.

2. The rise of serum protein, chloride and total fixed base with treadmill exercise, and their decrease with swimming have been shown to be due largely to changes in the water content of the body.

3. The relatively larger increase of serum protein concentration over those of chloride and total fixed base which was observed in the case of some of the dogs has been attributed to increase of either the hydrostatic pressure within the blood system or the osmotic pressure of the tissue cells during exercise, causing a transfer of fluid containing diffusible ions from the blood system to the interstitial fluids.

4. By means of a few assumptions, the changes which occur in the water, chloride and total fixed base contents of the red cell, serum and extracellular fluid phases of the organism of the dog during exercise and recovery have been calculated from cell and serum data.

5. Loss of water from the red cell, serum and extracellular fluid phases has been shown to be approximately 50 per cent of the loss in body weight which occurred during exercise.

6. A decrease in the base to chloride ratio of the serum, which cannot be explained by the chloride shift between cells and serum with shift of pH, has been accounted for largely as the result of loss of base from the system due to excretion by the kidneys of base with anions other than chloride, notably sulphate and phosphate.

REFERENCES

(1) Van Slyke, D. D. and J. M. Neill. J. Biol. Chem. **61**: 523, 1924.
(2) Hastings, A. B. and J. Sendroy, Jr. J. Biol. Chem. **61**: 695, 1924.
(3) Laudet, M. M. J. de pharmacol. et chim. **16**: 168, 1917.
(4) Stadie, W. C. and E. C. Ross. J. Biol. Chem. **65**: 735, 1925.
(5) Fiske, C. H. J. Biol. Chem. **51**: 55, 1922.
(6) Greenberg, D. M. J. Biol. Chem. **82**: 545, 1929.
(7) Pregl, F. and H. Roth. Quantitative organic microanalysis. 3rd Eng. ed., p. 86, P. Blakiston's & Sons., Philadelphia, 1937.
(8) Keys, A. and H. Taylor. J. Biol. Chem. **109**: 55, 1935.
(9) Dill, D. B., J. H. Talbott, and H. T. Edwards. J. Physiol. **69**: 267, 1930.

(10) HARRISON, H. E., D. C. DARROW AND H. YANNET. J. Biol. Chem. **113**: 515, 1936.
(11) PETERS, J. P., H. A. BULGER, A. J. EISENMAN AND C. LEE. J. Biol. Chem. **67**: 175, 1926.
(12) PETERS, J. P. AND D. D. VAN SLYKE. Quantitative clinical chemistry. Vol. 1, p. 957. The Williams & Wilkins Company, Baltimore, 1931.
(13) BRIGGS, A. P. Proc. Am. Soc. Biol. Chem. **8**: No. 6, xv, 1937; J. Biol. Chem. **119**: 1937.